# nature medicine

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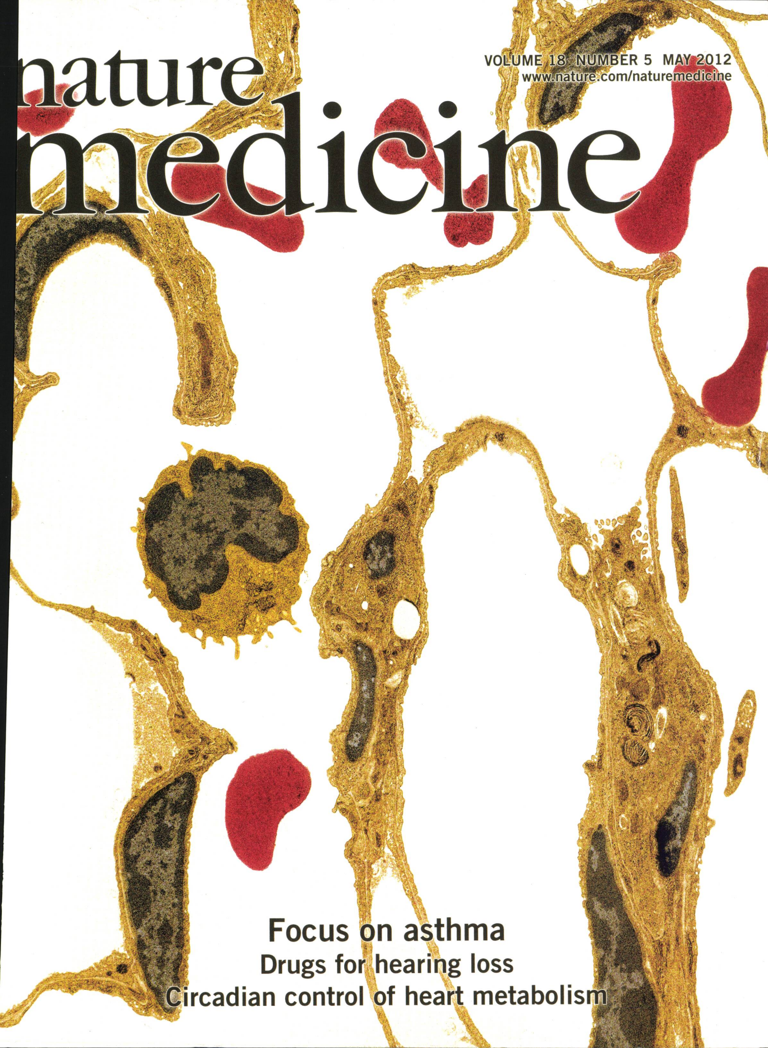

## Focus on asthma

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Circadian control of heart metabolism

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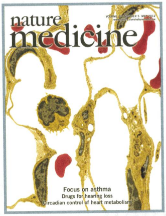

This month we feature a collection of reviews on asthma. The cover shows a pseudocolor electron micrograph of a mouse lung. Image courtesy of Thomas Deerinck and Mark Ellisman, NCMIR, University of California–San Diego.

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ZEYNE
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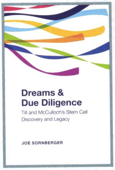
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Till and McCulloch's Stem Cell Discovery and Legacy
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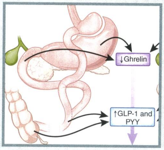

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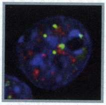

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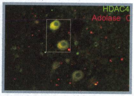

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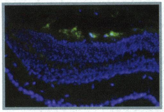

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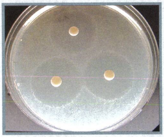

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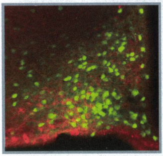

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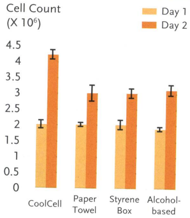

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# A fresh perspective on asthma

**Recent advances in asthma research have altered views of how the disease is triggered and sustained. What remains to be seen is whether this knowledge can be integrated into the clinic to alter the natural course of disease.**

The global prevalence of asthma has markedly increased over the past 50 years, with an estimated 300 million people affected worldwide. Following the description of IgE and the T helper type 1 ($T_H1$)/$T_H2$ hypothesis, scientists made considerable headway identifying pathways necessary to initiate and sustain allergic responses. However, clinical trials targeting these pathways met with mixed results, and, despite symptomatic control of the disease, individuals still develop chronic disease and remain refractory to therapies.

To identify recent advances that have affected understanding of asthma, *Nature Medicine* reached out to 50 basic and clinical researchers in asthma and asked them to highlight key advances and obstacles in the field. These recent developments are discussed in depth in seven reviews in this issue of *Nature Medicine*. We are indebted to the authors of these reviews and to the investigators surveyed for their insight into the field. What emerges is a picture of a highly heterogeneous disease, influenced by complex genetic and environmental effects, and an evolving understanding of the key cell types and soluble mediators that orchestrate the immune response.

One area of interest involves the intersection of microbial exposure with allergic disease, particularly as an early conditioning event in children. Although viral infections are known to exacerbate asthma, in a recent epidemiological study (*N. Engl. J. Med.* **364**, 701–709, 2011), children who grew up on farms were exposed to a larger diversity of microbes and were protected from asthma. Though the specific microbes conferring protection were not identified, the culprits may lie within the gut. Commensal bacteria in the intestine can influence the development of several immune cells known to influence allergy, including basophils, invariant natural killer cells and T cells. Clinically, these findings dovetail with epidemiological studies linking disruptions in the gut microbiome with increased risk of allergic airway disease. Moreover, recent metagenomic analyses have revealed that the density and composition of commensal bacteria in the airways differ between subjects with asthma and control subjects (*PLoS One* **5**, e8578, 2010). Though the role of these bacteria in asthma is unclear, these findings confirm the importance of early life exposure to microbes on the development of the immune system and allergic disease, and may point to a window of opportunity for intervention.

Epithelial cells lining the airways are the first line of defense against the entry of microbes and allergens. It is now clear that epithelial cells are not just passive participants responsible for walling off the body from the environment; instead, they respond to external cues and are directly activated by allergens through receptors of the innate immune system, releasing soluble mediators that orchestrate the subsequent adaptive immune response. These findings have overturned the idea that immune cells are the primary triggers of asthma and suggest that the airway epithelium has a sentinel role in bridging innate and adaptive immunity. Genetics studies have confirmed the importance of epithelial cells, as mutations in several epithelial cell–specific genes are associated with allergy and asthma. At present, it's unclear whether targeting innate receptors on epithelial cells can prevent the development of asthma in adulthood and what effect this might have on development of the immune system if initiated early in life.

Although epithelial cells are a source of soluble mediators, a burgeoning area of research concerns the identification of other sources of cytokines that promote $T_H2$ responses. Several newly identified innate subsets in the gut and lung produce cytokines and can contribute to allergic responses. The challenge now is to dissect their relative importance in relation to the complex array of adaptive immune cells in the lung (such as $T_H17$, $T_H9$ and regulatory T cells) that affect disease.

Nowhere is this complexity more apparent than in the clinic, as asthma does not present as a single disease entity. Rather, distinct phenotypes of asthma are identifiable, and these distinct cohorts respond differently to medications, a fact that may account for the recent failure of several targeted biologics that were tested in large, unselected patient populations. In a recent study, patients expressing higher serum concentrations of periostin, a protein released with activation of the IL-13 pathway, responded favorably to an interleukin-13–targeting antibody (*N. Engl. J. Med.* **365**, 1088–1098, 2011). The hope is that, in the future, biomarkers reflecting activation of a pathogenic cascade may be used to stratify individuals before treatment to increase the likelihood of success. By combining unbiased genetic approaches and immunophenotyping of individuals, asthma may eventually be subcategorized into distinct endotypes on the basis of molecular mechanisms, opening the door to personalized therapy. The existence of multiple or mixed endotypes suggests that combination therapy may be needed to adequately control disease in subsets of individuals with asthma.

These and other advances have challenged previously held hypotheses regarding the origins of disease and are fostering new avenues of research. The newly uncovered targets suggest novel ways of intervening early, curbing the progression to chronic disease and managing disease in an underserved population. Despite setbacks in recent clinical trials, we are confident these findings will add to the armamentarium in the near future.

In closing, we thank Almirall and Theravance for their financial support to produce this special issue of the journal. As always, *Nature Medicine* takes full editorial responsibility for the content of these pages. ■

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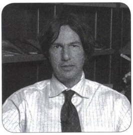

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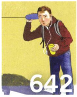
**Airing concerns:**
Critics question impact of malaria drug subsidy program

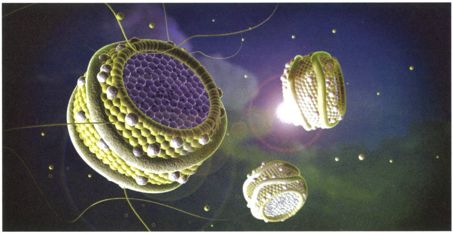
**Psore point:**
New biologic drugs for psoriasis help quell the immune system



**Hearing test:**
Drugs to prevent hearing loss enter clinical development

# Hopes soar as cholesterol plummets with new drug class

Statin drugs such as the blockbuster Lipitor (atorvastatin) typically reduce the levels of artery-clogging cholesterol in the bloodstream by 30–40%, with comparable reductions in the risk of cardiovascular disease in most people. But other interventions are needed to further drive down the amount of this 'bad' cholesterol, also known as low-density lipoprotein (LDL), in individuals at the highest risk of developing heart attacks and stroke. Now, researchers have published the first data from human trials showing that a combination of statins and new drugs directed against a key regulator of cholesterol metabolism can help bring people's cholesterol levels into the long-sought sweet spot.



3D4Medical / Photo Researchers, Inc.

**Statin alive:** Adding new medicines could kickstart current cholesterol drugs.

"These agents are achieving LDL reductions that you don't even see at the highest dose of statins," says cardiologist Evan Stein, director of the Metabolic and Atherosclerosis Research Center in Cincinnati, Ohio. "So they are a real alternative for patients who can't tolerate statins and give substantial additional reductions for patients who are maxed out on statins and still not reaching target—and that's a lot of patients."

The leading drug in this new class is a monoclonal antibody that Stein is investigating. In March, Stein and his colleagues reported phase 1 data showing that the drug—REGN727, which is being co-developed by Regeneron Pharmaceuticals of Tarrytown, New York and the French drug giant Sanofi—reduced blood levels of LDL cholesterol by up to 61% compared with placebo in people concurrently taking Lipitor (*N. Engl. J. Med.* **366**, 1108–1118, 2012). A few days later, at the American College of Cardiology meeting in Chicago, a team that included Stein presented the results of a 183-person, phase 2 trial of REGN727 showing a similarly steep reduction in cholesterol concentrations with only one serious adverse event (*J. Am. Coll. Cardiol.* doi:10.1016/j.jacc.2012.03.007, 2012).

That same week, Thousand Oaks, California–based Amgen also announced phase 1 data for a similar agent, dubbed AMG 145, which reduced average cholesterol levels by up to 75% in study subjects on stable doses of statins.

Both the Amgen and Regeneron-Sanofi drugs work by inhibiting an enzyme called proprotein convertase subtilisin/kexin type 9 (PCSK9), which normally destroys LDL receptors on the surface of liver cells and thereby makes it hard for those cells to remove cholesterol from the blood. Statins, in contrast, work mainly by inhibiting an enzyme that helps make cholesterol; unfortunately, they also increase the production of PCSK9, which attenuates their LDL-lowering potential.

## Different strokes for different folks

Amgen is currently launching several phase 2 trials of AMG 145, whereas Sanofi and Regeneron are recruiting for a pivotal phase 3 study of REGN727. Several other large companies, including Pfizer, Merck and Novartis, also have PCSK9-directed antibodies in or nearing phase 1 development. But not every drugmaker is using the same toolkit to go after PCSK9. A number of smaller firms also have experimental antisense therapies in early clinical or late-stage preclinical development.

Last month, Alnylam Pharmaceuticals of Cambridge, Massachusetts announced updated results from a 32-person trial testing an RNA interference–based therapeutic that reduced LDL levels in the blood by up to 50%. And in the February issue of *Molecular Therapy* (**20**, 376–381, 2012), Marie Lindholm and her colleagues at Santaris Pharma in Denmark published monkey data showing a comparable silencing technique involving locked nucleic acid–modified oligonucleotides cut cholesterol levels in half.

Santaris's human trials to date have proven lackluster, though, and in October of last year the company halted clinical testing owing to poor efficacy data. "We are currently looking to bring forth a backup candidate with a better therapeutic index," Lindholm says. Similarly, in 2010, New Jersey's Bristol-Myers Squibb and California's Isis Pharmaceuticals stopped a phase 1 study of another PCSK9-targeted antisense therapy, and the two drugmakers are now working to advance a more potent antisense inhibitor.

However antisense approaches ultimately fare, antibodies will probably be the first to market, with the drugs projected to generate billions of dollars in revenue for their manufacturers. Yet with several top-selling statins, including Lipitor, now available as cheap generics costing as little as $1 per day, analysts worry over whether people will embrace the PCSK9-directed antibodies, which, if approved, will probably be priced at $20,000 or more per year.

"The potential choice might boil down to dirt-cheap generic statin or dirt-cheap generic statin plus PCSK9 antibody," says Mike Mitchell, a healthcare analyst at Seymour Pierce, a London-based investment bank. "Suddenly, the economic profile of altering an individual's LDL changes dramatically."

*David Holmes*

# Malaria subsidy pilot soars, but some see turbulence ahead

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**Private matters:** A private pharmacy in Tororo, Uganda sells AMFm-subsidized drugs.

Amy Maxmen

KAMPALA, UGANDA — In 2010, a Kenyan drug importation and distribution company named Harley's Limited stopped ordering low-cost, mediocre malaria medications, and began ramping up its orders of high-quality ones—by about fourfold. Vinod Guptan, head of marketing at the Nairobi-based company, says demand shifted when the price of the best drugs dropped, making them cheaper for vendors, who passed the savings on to local consumers. The about-face in preference illustrates the strong influence of the Affordable Medicines Facility for malaria (AMFm), a radical program designed to shift the market for malaria drugs that now faces scrutiny for wielding too much force.

A team convened by the US Institute of Medicine (IOM) developed the idea for the effort in 2004, when malaria morbidity rates were rising in developing countries in part because many people with the disease opted for chloroquine, despite widespread resistance to the decades-old drug, or for rudimentary 'monotherapy' treatments that contained only a single active ingredient, artemisinin. The group reasoned that patients would switch to the leading artemisinin-based combination therapies (ACTs) if they cost less than the alternatives at drug shops because of subsidies offered to importers at the top of the supply chain.

"The fact is that these are poor countries with poor public health systems," explains Kenneth Arrow, an economist at Stanford University in California and an author on the IOM report. "When I discovered that 70% of chloroquine was distributed commercially, I immediately came to the conclusion that ACTs would have to be distributed through the private sector if we wanted nearly universal coverage."

After much discussion, the AMFm was born as a three-year pilot program in 2010 under the auspices of the Geneva-based Global Fund to Fight AIDS, Tuberculosis and Malaria. Support for the first phase totaling $316 million came from the Seattle-based Bill & Melinda Gates Foundation, the UK's Department for International Development and money raised mainly through airline ticket fees by a UNITAID partnership with nine countries.

In providing co-payments for drug distributors in the private as well as the public domain, the AMFm represents the first large-scale medical aid initiative to partner directly with the unregulated commercial sector in sub-Saharan Africa, says AMFm Director Olusoji Adeyi. Traditionally, international organizations help the government-run public sector obtain ACTs, which they then provide for free or nearly free. In many countries, however, villagers purchase drugs from shops when public clinics are closed or far away or have no medicines in stock.

Now in the final year of its pilot phase, AMFm has altered the malaria drug distribution landscape. But some global health coordinators voice concerns about how dramatically the initiative has altered the supply chain and funneled ACTs into just a handful of the countries that need them. The scrutiny comes amid an independent evaluation of AMFm contracted by the Global Fund and conducted by ICF International, a Fairfax, Virginia–based consultancy, and the London School of Hygiene and Tropical Medicine. Launched in February 2010, the assessment will inform a decision about AMFm's future in November or December of this year.

## The price is right

Inadequate malaria medications no longer cost far less than ACTs in at least six of the eight countries selected for AMFm support: Ghana, Kenya, Madagascar, Nigeria, Tanzania (including Zanzibar) and Uganda. Before the initiative, vendors sold ACTs for between $6 and $11. Now, at roughly $1 per dose, the drugs are cheaper than monotherapy and about the same price as chloroquine. "No one even wants those others anymore because ACTs work rapidly and cost the same," says Guptan. "In 2009, we used to sell around 15,000 to 20,000 ACT [treatment packs] a month, and by the end of 2011 the orders were up to 200,000."

Last year, about 60% of the world's estimated supply of ACTs was allocated through AMFm channels, *Nature Medicine* has learned. By August, aid organizations had begun to complain of delays. The Swiss pharmaceutical company Novartis informed one of the largest malaria programs, the US President's Malaria Initiative (PMI), that it could not quickly fill a request for 18 million treatment packs. Some nations, such as Somalia, needed the doses urgently before the rainy season began. (Rain creates puddles from which swarms of mosquitoes, the vectors for malaria, hatch.)

"There was a clear rush last year between March and July of big orders from AMFm," explains Andrea Bosman, a malaria program coordinator at the World Health Organization (WHO) in Geneva. "Several funding agencies signaled that manufacturers were unable to meet their demands, and in some cases the manufacturers could not accept new orders. We were really worried."

At the heart of the ACT supply issues lies the reliance on artemisinin, a natural compound derived from sweet wormwood *Artemesia annua,* which farmers must plant ten months before drug manufacturers can use it. Predicting artemisinin demand has never been simple, and last year manufacturers were unprepared for an onslaught of requests from AMFm-subsidized buyers, explains Adeyi. Unlike orders from aid organizations and governments often bogged down in bureaucracy, Adeyi says the private sector ordered drugs efficiently. "What we have demonstrated, is that products move far more swiftly in the commercial sector than in the public sector," he says. "But there is some ideological discomfort with that among global health practitioners because of their tradition of favoring public-sector channels."

**Where do artemisinin-based combination therapies go?**

| | |
|---|---|
| 98 million (treatments) | AMFm (private-sector orders delivered in 2011) |
| 92 million | Global Fund (public sector, estimated procurement in 2011) |
| 69 million | Non–Global Fund, non-PMI public sector (estimated 2011 procurements) |
| 32 million | PMI (estimated procurement in 2011) |
| 8 million | Full-price private sector (estimated demand in 2011) |

- AMFm 2011 data are from 11 January 2011 to 30 December 2011. The AMFm private-sector figure includes both the private for-profit and private not-for-profit deliveries, but excludes AMFm-funded public-sector deliveries and those delivered in Cambodia, which have been included in the premium private-sector estimate.
- Global Fund numbers include AMFmfunded public sector deliveries.
- Non–Global Fund numbers include the World Bank, the UK Department for International Development and others.
- PMI numbers here have been adjusted for 2011.
- All public-sector numbers (PMI, Global Fund, others) have a 25% disbursement delay factor applied to them.

ACT market data from Prashant Yadav, William Davidson Institute at the University of Michigan

### Delivering promises

In response to the turmoil last summer, the WHO held a meeting in Geneva on 8 September 2011 with representatives from relevant organizations and drug companies—including the Global Fund, PMI and Novartis. Ultimately, the group averted disaster by shifting orders among manufacturers, allocating donor funds from one country to another and requesting that the AMFm slows its approvals for subsidies. "We learned that there wasn't a worldwide shortage," says Bosman, "but that the balance between supply and demand is very tight."

Still, delays persist, says Jean-Marie Kindermans, an administrator in Paris at the nonprofit Doctors without Borders, which stocks ACTs at its clinics in 30 malaria-endemic countries. Kindermans says that, in early 2011, Novartis shipped packages of the common ACT coartem within five weeks. Now the drugs take at least two months to arrive.

Beyond these delays, some people find it disconcerting that so much of the ACT supply goes to AMFm countries even though they represent about a quarter of the countries in which more than 1,000 people die of malaria each year. "Some AMFm countries, like Madagascar, aren't ones with high endemicity," Kindermans says. Of 43 African countries with malaria, at least 26 have more cases than that island state. And Ghana, with approximately 2.6 million malaria cases, was chosen by AMFm rather than Burkina Faso, the Democratic Republic of Congo or Malawi, which according to 2010 WHO figures struggle with twice that number.

Critics also worry that some of the ACTs handled by private sector vendors were sold to consumers regardless of whether the buyers had a diagnosis. "Buyers in Zanzibar—an island where malaria is almost eliminated—ordered 241,000 AMFm-subsidized doses," says Mohga Kamal-Yanni, a health advisor in the UK at the international nonprofit Oxfam. "Given that this medicine has a short shelf life, will all of these ACTs truly be sold to people with malaria? The figures don't tally, and, because of this demand, there are shortages elsewhere in the market."

### Leaning on levers

Now the AMFm applies a series of so-called 'levers' to rationally approve orders, making it unlikely that Zanzibar will receive AMFm-subsidized doses again, according to Adeyi. "We are learning lessons as we go," he says. To explore how the program would function, he explains that countries were chosen not only on the basis of their malaria burden, but also the countries' experiences with ACTs purchased through Global Fund grants, and the involvement of the private sector in drug deployment.

Some effects of the pilot were unexpected. For example, public hospitals in Ghana—which historically procure ACTs through the government and aid programs—have bought AMFm-subsidized drugs from private vendors. Adeyi says that this unprecedented phenomenon illustrates how much more efficient the AMFm system is. However, Kamal-Yanni worries that if public hospitals rely on the commercial sector, and prices go up, they'll be forced to cut parts of their health budget to buy ACTs.

Another consequence is that several local drug manufacturers have halted their operations, says James Tibenderana, technical director of the African branch of the Malaria Consortium partnership based in Kampala, Uganda. Rather than buying local, drug wholesalers prefer to purchase subsidized drugs from AMFm's list of seven permitted companies, only one of which resides in Africa. "ACT manufacturers outside of the AMFm are moving on to making other drugs, leaving fewer companies on the market," explains Tibenderana. "So if AMFm comes to an end, the only players that will be capable of providing ACTs are the ones who had been included in the program."

Larger questions loom over whether the program will continue, expand or terminate next year. Growers of sweet wormwood in China needed to know how many seeds to plant last November, but the official answer from the Global Fund about the fate of the program won't be out until this December. "The fact that so many people are left in the dark like this, I guess to me, that is what's most shocking," Bosman says.

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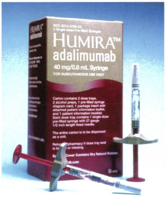
**Combination of factors:** Prices and availability affect consumer choice of malaria drugs.

# Biologic drugs set to top 2012 sales

Move aside, small-molecule drugs for common diseases. Three biologic medicines initially developed to treat rheumatoid arthritis—a joint disorder that afflicts just 1% of the population worldwide—are on track to top the sales charts this year. According to forecasts compiled by Thomson Reuters's pharmaceutical intelligence unit, Humira (adalimumab) and two comparable antibodies that also target tumor necrosis factor-alpha, a cytokine involved in rheumatoid arthritis, will lead the list.

The trio will topple the cholesterol drug Lipitor (atorvastatin) and the blood thinner Plavix (clopidogrel) to become the world's top-selling medicines in 2012. Despite the smaller patient populations targeted by these biologics, the higher prices they demand can still translate into a sizeable market share, explains analyst Saurabh Aggarwal, principal of Novel Health Strategies, a life sciences consulting firm based in Bethesda, Maryland. "The pricing is a big factor that's driving the high sales in these products," he says.



Abbott Laboratories

| Drug | Projected 2012 sales | Company | Primary indications | Drug type |
|---|---|---|---|---|
| Humira (adalimumab) | $9.3 billion | Abbott Laboratories | Rheumatoid arthritis and psoriasis | Antibody |
| Remicade (infliximab) | $9.1 billion | Johnson & Johnson | Rheumatoid arthritis and psoriasis | Antibody |
| Enbrel (etanercept) | $8.1 billion | Amgen | Rheumatoid arthritis and psoriasis | Antibody |
| Advair (fluticasone/salmeterol) | $8.0 billion | GlaxoSmithKline | Asthma and chronic obstructive pulmonary disease | Small molecule |
| Rituxan (rituximab) | $7.1 billion | Genentech/Roche | Blood cancers and rheumatoid arthritis | Antibody |
| Crestor (rosuvastatin) | $7.0 billion | AstraZeneca | Cardiovascular disease | Small molecule |
| Avastin (bevacizumab) | $6.1 billion | Genentech/Roche | Various cancers | Antibody |
| Herceptin (trastuzumab) | $6.1 billion | Genentech/Roche | Breast cancer | Antibody |
| Lantus (insulin analog) | $5.9 billion | Sanofi | Diabetes | Protein |
| Abilify (aripiprazole) | $5.9 billion | Bristol-Myers Squibb | Schizophrenia, bipolar disorder and depression | Small molecule |

Source: Thomson Reuters Pharma

# Targeting hypoxia brings breath of fresh air to cancer therapy

Solid tumors are often starved of oxygen, as new blood vessel formation typically can't keep pace with rapid cell division. Yet, rather than suffocating cancer tissue, this 'tumor hypoxia' usually renders cancer cells resistant to standard therapies, as oxygen is necessary to fuel the development of DNA strand breaks in radiation therapy and the dearth of blood vessels keeps circulating chemotherapy regimes away. Even with this elemental problem, however, some changes in hypoxic cells could actually result in increased drug sensitivity—a weakness that many drug developers hope to exploit for a next generation of tailored cancer treatments.

Leading the pack of so-called 'bioreductive prodrugs'—agents that become activated only in the oxygen-poor core of solid tumors—is Threshold Pharmaceuticals' TH-302. In a talk presented on 2 April at the American Association for Cancer Research's annual meeting in Chicago, Threshold announced the results of a phase 2 trial involving 214 people with advanced pancreatic cancer. Compared with chemotherapy alone, the addition of TH-302 increased the average progression-free survival time from 3.6 to 5.6 months, the San Francisco–based drugmaker found.

"We are excited about the results," says Stewart Kroll, head of biostatistics and clinical operations at Threshold. "They have provided the proof of concept in a very difficult-to-treat indication."

These findings come on the heels of another phase 2 trial reported at last year's Connective Tissue Oncology Society meeting, also in Chicago, showing that TH-302 boosted the expected survival rates in people with soft-tissue sarcoma, a cancer of the body's connective or supportive tissues such as muscle, cartilage or fat. In February, Threshold announced that it was teaming up with Merck KGaA, the German pharmaceutical company, to further develop and commercialize TH-302 for a range of cancers; and in March, the agent was granted orphan drug status in the US and Europe to treat soft-tissue sarcoma.

The road leading to drugs such as TH-302, however, has been long and rocky. "Designing prodrugs for hypoxia turns out to be a difficult challenge," says Bill Wilson, a cancer biologist at the University of Auckland in New Zealand and the cofounder of Proacta, a San Diego–based company developing hypoxia-activated agents. "The target cells are distant from the systemic circulation, so they present a difficult drug delivery problem."

Two years ago, for example, a promising drug candidate called tirapazamine failed in a phase 3 trial involving more than 800 people with head-and-neck cancer, in part because the agent, made by SRI International, a California-based nonprofit research institute, was activated by enzymes in oxygen-rich regions and therefore used up before it reached the critical hypoxic cancer cells. Similarly, in April, Nevada-based Spectrum Pharmaceuticals announced that its drug apaziquone failed to meet primary endpoints in two phase 3 trials in patients with bladder cancer, but those results proved statistically

# Billions of dollars for research at stake in health-reform case

In April, shortly after the US Supreme Court had wrapped up three days of hearing arguments regarding the constitutionality of the country's 2010 Patient Protection and Affordable Care Act (PPACA), President Barack Obama noted that without the law "you cannot have a mechanism to ensure that people with preexisting conditions can actually get health care." But concerns as to the fate of the law go beyond hospital care. Several newly created biomedical research agencies and billions of dollars in funding will take a blow if the Supreme Court strikes down not only the mandate that individuals purchase insurance but possibly the entirety of the more-than-2,000-page law.

"Researchers ought to be concerned about the PPACA because the controversy could seriously hamper the progress we've made in the last few years," says Jerry Krishnan, who teaches medicine and public health at the University of Illinois College of Medicine at Chicago and also chairs the US Food and Drug Administration's pulmonary drug advisory committee.

The parts of the law affecting research include a rule stating that, beginning next year, providers must disclose research contracts to patients, including those with pharmaceutical companies, and a mandate that, from 2014 forward, insurers must cover clinical trial participation for beneficiaries of their health plans. Without the latter, many patients may lack the opportunity to participate in clinical trials and may lack access to experimental, but sometimes life-saving, medicine. The new law also created the Cures Acceleration Network, a grant program within the US National Institutes of Health (NIH) that, dependent on appropriations through Congress, could fund up to $500 million in projects per year to turn basic scientific discoveries into treatments. In addition, the PPACA pays particular attention to breast cancer, directing the NIH to conduct research on new screening methods, prevention and early detection of the disease in young women.

Funding of the Patient Centered Outcomes Research Institute—a nongovernmental body based in Washington, DC designed to improve personalized health care through understanding variation in treatment responses—also hangs in the balance. The PPACA established a funding stream for the institute that would dedicate up to $4 billion over ten years. Experts applaud the comparative effectiveness efforts that this institute will identify and reject the notion that it will stifle research. "Some believe that will deter pharmaceutical companies from making 'me too' drugs, but do you really think a manufacturer is not going to invest a billion dollars in doing something new?" says Freeman Farrow, a health law expert at DePaul University College of Law in Chicago.

There is worry, however, that if the entire PPACA is struck down, certain regulatory structures will be thrown into disarray. For example, the PPACA allows for generic versions of biologic drugs after 12 years of patent protections—a move that the industry supported, because the Obama administration had originally proposed shortening the exclusivity period to just seven years. And healthcare reform imposes up to $4 billion in new fees on drug and device manufacturers through 2018. "Of course, manufacturers also stand to gain millions of dollars in new revenue as a result of the expansion of coverage," says Topher Spiro, managing director for health policy at the Center for American Progress, a left-leaning think tank in Washington, DC.

A decision by the Supreme Court on the case, including whether the PPACA is an unconstitutional expansion of congressional power under the federal interstate commerce clause, is expected in June.

*John Otrompke*

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favorable when the data were pooled. The company is now seeking a meeting with the US Food and Drug Administration to discuss the next steps.

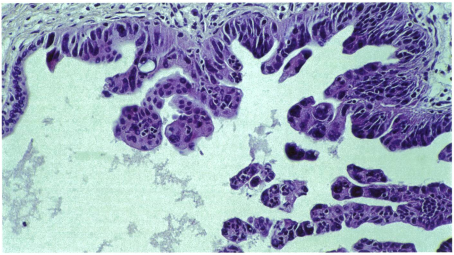
## Going pro

Other cancer-killing prodrugs moving forward include Proacta's PR-104 and Allos Therapeutics' RH-1. Similar to TH-302, these drugs are converted into toxic molecules in hypoxic cells to make powerful DNA-damaging agents; oxygen inhibits this process, leaving most healthy cells untouched. Proacta is currently recruiting participants for a phase 2 study involving people with acute myeloid leukemia, and Colorado's Allos—which was acquired last month by Spectrum—recently finished a phase 1 trial testing its drug in subjects with advanced solid malignancies.

According to Martin Brown, a radiation oncologist at the Stanford University School of Medicine in California and a founding scientist at Proacta, these prodrugs could be used to treat "a very wide spectrum of tumors." However, he cautions that "there's absolutely no point in giving these drugs, which are bound to have some toxicities, to patients who don't have hypoxic tumors."

To that end, Wilson and his colleagues have been looking for molecular biomarkers that might help identify the people most likely to benefit from hypoxia-targeted therapy. Earlier this year, his team showed that a compound called 2-nitroimidazole EF5, which is currently in clinical development as a signpost of hypoxia, also flagged the enzymes that convert prodrugs into their active forms (*Clin. Cancer Res.* **18**, 1684–1695, 2012). "We are making real progress," Wilson says, "and the field is poised for major advances in the near future."

*Melinda Wenner Moyer*



Parviz M. Pour / Photo Researchers, Inc.

**Elemental solution:** Threshold Pharmaceuticals' drugs trialed for pancreatic cancer (shown here).

# New biologic drugs get under the skin of psoriasis

New results on the clinical efficacy of two experimental psoriasis medications are creating a buzz among those who study and treat the skin disease. The drugs—ixekizumab from Indiana-based Eli Lilly and brodalumab from California's Amgen—are the first to exclusively target an immune signaling molecule known as interleukin-17 (IL-17) and its receptor. As such, new phase 2 data from both agents validate IL-17 as vital to psoriasis and offer the possibility of treatment for people who have failed existing therapies for this autoimmune disease.

"This patient population has had precious few rationale drugs over the decades," says Craig Leonardi, a dermatologist at St. Louis University School of Medicine and an author on the new studies of both ixekizumab and brodalumab, published simultaneously in late March. "I usually refer to the old days as the therapeutic wasteland. We were using tar coal, ultraviolet light and medicines with very significant toxicities. It's been an amazing renaissance to get where we are now."

In psoriasis, the body misidentifies skin cells as pathogens and sends T cells from the immune system to the skin, where they release cytokines, chemicals that activate inflammation pathways. The constant production of new skin cells causes red and white patches to form on surface; in some individuals, the internal inflammation associated with the condition can contribute to arthritis in the joints.

Existing biologic drugs on the market, including Humira (adalimumab) from Chicago-based Abbott Laboratories and Enbrel (etanercept) from Amgen, mostly target the cytokine known as tumor necrosis factor alpha, a major instigator of inflammation (see 'Biologic drugs set to top 2012 sales' on page 636). But over time the drugs can stop being as effective, and they can cause liver or gastrointestinal problems. More recently, however, companies have begun to develop monoclonal antibodies revolving around type 17 helper T cells ($T_H$17 cells), found in high levels in psoriatic lesions.

The most recent such psoriasis biologic to hit the market, Stelara (ustekinumab), made by Pennsylvania-based Janssen Biotech, blocks the protein domain p40 within two molecules, including interleukin-23, a cytokine that would otherwise promote the production of $T_H$17 cells. But the cells have some useful functions and blocking them entirely might end up being tough on the body in the long run. So other antibody-based therapies on the horizon block individual cytokines produced by the $T_H$17 cells, with the aim of producing fewer side effects and long-term toxicity.

## The upside of downstream

Ixekizumab and brodalumab both work by stopping the production of IL-17 cytokines by $T_H$17 cells. "They're working on the same cascade of events but hitting it downstream," explains Leonardi.

In the trial of ixekizumab, 142 patients received either a placebo or one of four different doses of the drug injected into the skin. After three months, 82% of patients receiving the highest two doses of ixekizumab saw a reduction of at least 75% in psoriasis severity according to a common psoriasis scoring system; by comparison, less than 8% of those taking the placebo saw such improvement (*N. Engl. J. Med.* **366**, 1190–1199, 2012). In the similarly designed three-month trial of brodalumab, 198 patients received placebo or four doses of the drug. Around 86% of those taking the highest doses saw a 75% improvement in their disease, compared with 3% of those taking placebo (*N. Engl. J. Med.* **366**, 1181–1189, 2012).

Mehau Kulyk / Photo Researchers, Inc.

**Waving goodbye:** Targeting IL-17 could be the key.

Notably, both drugs work in about 10–20% more people than do the existing drugs, an exciting prospect for those who fall into the group of people unable to find psoriasis treatments that work. "The data shown in both the new papers have really outstanding numbers for efficacy, so I think what needs to be determined with these drugs is their long-term safety," says Alice Gottlieb, a dermatologist at the Tufts Medical Center in Boston who has studied Stelara and briakinumab, a similar experimental psoriasis antibody from Abbott Laboratories.

Because of the new biologics in development, analyst Bingnan Kang of Decision Resources, a Burlington, Massachusetts–based market research firm, expects the US psoriasis market to grow from \$3.9 billion in 2010 to more than \$7.4 billion by the end of the decade. But exactly which drug—ixekizumab, brodalumab or any of the other interleukin-directed agents in mid- to late-stage clinical development (see 'Rolling in the ILs')—will end up ahead is hard to predict, notes Kang, especially with such similar efficacy numbers coming out of the recent trials.

"The initial uptake will always be slow in the psoriasis market," she says, "and more information on long-term safety is really essential right now to convince physicians to switch to a new drug."

Ultimately, though, there are still needs for others types of psoriasis treatments—for both people whose psoriasis is not severe enough to warrant treatment with biologics and those who have more severe cases, Gottlieb points out. "There are needs in psoriasis that the biologics don't meet," she says. "A great unmet need is better topical treatments and also safer and more effective oral medications. Psoriasis is by no means a done deal."

*Sarah C P Williams*

**Rolling in the ILs**: Interleukin-directed antibodies in development for psoriasis.

| Drug | Company | Target | Phase |
|---|---|---|---|
| Stelara (ustekinumab) | Janssen Biotech | IL-12/IL-23 (p40 subunit) | Approved |
| Briakinumab | Abbott Laboratories | IL-12/IL-23 (p40 subunit) | 3 |
| Ixekizumab | Eli Lilly | IL-17 | 2 |
| Brodalumab | Amgen | IL-17 receptor | 2 |
| Secukinumab | Novartis | IL-17 | 2 |
| Fezakinumab | Pfizer | IL-22 | 2 |
| MK-3222 | Merck | IL-23 (p19 subunit) | 2 |
| CNTO 1959 | Janssen Biotech | IL-23 (p19 subunit) | 2 |

National Foundation for Cancer Research

# Straight talk with…
# Chen Zhu

Before becoming China's Minister of Health in 2007, Chen Zhu had witnessed a wide contrast in medicine within his homeland. When he was sent to the countryside and worked as a 'barefoot doctor' during the Cultural Revolution, he saw the need for basic care in rural regions. As a researcher, he helped reveal the mechanism through which all-trans retinoic acid and arsenic trioxide—a compound used in traditional Chinese medicine for more than two millennia—can radically improve standard care for acute promyelocytic leukemia (APL).

In April, Chen and his mentor, Wang Zhen-yi of the Shanghai Jiao Tong University School of Medicine, received the Albert Szent-Györgi Prize, a $25,000 cash award from the Washington, DC–based National Foundation for Cancer Research, in recognition for their work on APL. ON that occasion **Victoria Aranda** and **Roxanne Khamsi** asked Chen about his plans for cancer research and for improving stem cell regulation in China. A condensed version of that interview follows.

**What does the example of APL teach us about translational medicine?**
After the clinical success [with APL], we were interested in the molecular and cellular mechanism of the treatment: differentiation induction therapy. Arsenic trioxide works on the patients who have relapsed after retinoic acid, suggesting that [the molecules work through] different mechanisms. After many years of work, we realized that if we are talking about targeted therapies, then this is typical of synergistic targeting.

I think that if we go back to history, then first of all there are different approaches to treating cancers. Killing—chemotherapy, a cytotoxicity approach—is still a useful approach, but, on the other hand, targeted therapies should also be very important, such as differentiation therapy. They must be targeting the leukemia stem cells; they must target the focal point involved in the leukemogenesis network

**How can we find targets to extend this idea, making malignant cells into benign cells, to other cancers?**
The contribution of cancer genomics can be to identify more targets. So, by combining both chemical biology and genomics and systems biology then in the future, more effective drugs or therapies should be able to be found.

**How do you envision incorporating personalized medicine in the clinic?**
The story of APL tells us that actually the use of biomarkers should be very important for the right person to choose the right drugs, because retinoic acid and arsenic work very well in APL but unfortunately not in other [types of leukemia]. [Sequencing for such mutations] is routine practice in some big cancer centers in China, already. In the future, we need to improve the guidelines for cancer diagnosis and treatment by using useful biomarkers as much as possible.

**How is your research in cancer informing your decisions about how much a drug should cost?**
It's a difficult question. As a minister of health with limited resources in a developing country of 1.3 billion [people], we should also be very prudent of the selection of drugs in our essential drug list. A very expensive drug [might] allow a prolongation of maybe just two or three months. I don't mean that we shouldn't consider the use of expensive drugs that allow just a few months of life. But, for example, in China we have so many children with major diseases, such as congenital heart disease or childhood leukemia. And for childhood leukemia, the treatment is very effective. Eighty percent of the children who have acute lymphocytic leukemia are curable, so I have to balance [that]. It's not easy.

**What do you see in terms of the challenges for drug manufacturing in China?**
We need to encourage innovation of the drugs, but I think we should pay more attention to the use of the generic drugs. The Chinese pharmaceutical industry is developing quite rapidly, particularly since the healthcare reform [of 2009], because the needs are there. Before [health care reform in China], many patients didn't see a doctor because of a lack of the means to do so. Now there are more and more people who go to the doctor when they are sick. That means there's a huge demand for medical services, but also a huge opportunity for the pharmaceutical industry.

**The US Food and Drug Administration is expanding its reach of monitoring drug manufacturing. What is China doing to make sure drugs are reliable?**
Of course quality should be the most important thing. China is upgrading the GMP [good manufacturing practice] standards for most of the drugs, particularly for the injectable drugs. Last year the new version of the GMP was issued; I'm sure that will allow a higher quality of the drugs [to be] manufactured in China. There's a period of three to five years [for implementation].

**What's your philosophy toward regulating stem cell therapies?**
I think stem cell therapy is still at the very beginning. Recently, the minister of health decided to reinforce the regulation on this. Not long ago, actually, we made a decision that we should revisit all the clinical trials for those hospitals that haven't yet got approval and that they should stop.

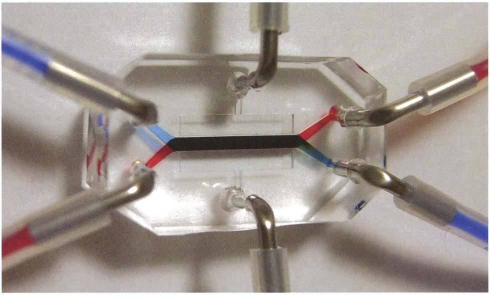
**When you come across an instance when there has been misconduct, in some cases the death penalty is used in China. Is that something that will continue in the future in the case of medicine?**
The death penalty is very rarely used in China for misconduct unless it concerns severe damage to human life. I think most importantly we need to stop the tendency [of criminals] of using new technologies to get money. This is the big issue.

# Biomedical briefing

## POLICY

### Flu turn

On 30 March, the US National Science Advisory Board for Biosecurity reversed its previous decision on two controversial flu studies and recommended that they should be published in their entirety. The two manuscripts, which had yet to be released as *Nature Medicine* went to press, describe ways to make the H5N1 avian influenza virus more transmissible between ferrets, a commonly used stand-in for humans in flu studies. In response to criticisms that the US National Institutes of Health (NIH) funded the research without considering its malfeasant potential, on 29 March the US government outlined a new policy that will require federal agencies to systematically review the potential risks of proposed projects involving 15 "high consequence" pathogens and toxins, including the avian flu and Ebola viruses and anthrax toxin. The policy went into effect immediately.



Wyss Institute

### Gut-on-a-chip mimics human intestine in miniature form

Here's one chip that might be good for your health. A team from Harvard University's Wyss Institute for Biologically Inspired Engineering in Boston has created a microfluidic device that mirrors the complex mechanics and physiology of the human gut—complete with villi-forming intestinal cells and living symbiotic bacteria (*Lab Chip* doi:10.1039/c2lc40074j, 2012). "It really does capture an aspect of *in vivo*–like structure and organization," says Arul Jayaraman, a bioengineer at Texas A&M University in College Station who was not involved in the research. "They not only have flow but the pulsatile motion that mimics the intestinal environment." The USB flash drive–sized device could allow researchers to model gut disorders such as Crohn's disease and to screen new drugs.

### Law of nature

The US Supreme Court issued a unanimous decision on 20 March that invalidated two medical diagnostics patents held by Prometheus Labs, a San Diego–based subsidiary of the Swiss food giant Nestlé. In a 24-page opinion, the court found that Prometheus's patents on methods for optimizing drug dosage on the basis of blood tests "effectively claim the underlying laws of nature themselves." The decision could have ripple effects throughout the biopharma industry—including, notably, in the lawsuit challenging the breast cancer susceptibility gene patents held by Salt Lake City's Myriad Genetics. On 26 March, the Supreme Court ordered an appeals court to reconsider that case. The Prometheus ruling "may make it more difficult to obtain a patent for personalized medicines, lessening the economic incentive to develop them," says Christopher Ohly, a patent and pharmaceutical lawyer at the firm Schiff Hardin in Washington, DC.

### Safety standard

The US Food and Drug Administration must share more safety inspection and training responsibilities with regulators in Europe and other developed countries, according to a report published last month by the US Institute of Medicine that calls for an international standard for drug surveillance. "If you look at the magnitude of drugs, the number of manufacturers and the complexity of the supply chains, there's just no way one agency can do it alone," says Jim Riviere, a pharmacologist at North Carolina State University in Raleigh and the report's lead author. See go.nature.com/7QzzoD for more.

## FUNDING

### Dementia deluge

Less than two months after US President Barack Obama pledged to significantly increase spending on research into Alzheimer's disease and its debilitating effects, his UK counterpart followed suit. On 26 March, Prime Minister David Cameron announced a "national challenge on dementia" and outlined plans to boost funding for research in this area from the £27 million ($42 million) spent in 2010 to £66 million annually by 2015. "UK investment in dementia research has long lagged behind levels invested in other serious diseases," says Shirley Cramer, chief executive of Alzheimer's Research UK, a Cambridge-based nonprofit, "but the prime minister's doubling of public funding for dementia research begins to address this."

### Wellcome venture

On 20 March, the Europe's largest biomedical research charity, the Wellcome Trust, launched a new venture capital unit that will invest in start-ups focused on both healthcare and the life sciences. The £200 million

($320 million) fund, known as Project Sigma, will initially back companies in Europe and then expand globally if initial investments prove fruitful. "The market needs this kind of fund," says Martin Murphy, a former partner at the London-based venture capital firm MVM Life Science Partners who is leading the new project. "I think we can deliver big returns." A week later, Cancer Research UK's commercial arm and a branch of the European Investment Bank also jointly launched a £50 million investment fund, and in April the UK government unveiled a £180 million scheme to revive the country's biotech industry.

## PEOPLE

### Banking on health

Global health expert Jim Yong Kim has been tapped as the next president of the World Bank. Kim, a doctor and medical anthropologist by training, had previously led the World Health Organization's HIV/AIDS unit and was a founder of Partners In Health, a Boston-based nonprofit group that provides medical care in developing countries. Since 2009, he has served as president of Dartmouth College in Hanover, New Hampshire, a position he will vacate on 1 July to take the helm of the Washington, DC–based development bank.

### PATH to health

On 26 March, the global health nonprofit PATH named Steve Davis, director of social innovation at the consulting firm McKinsey, as its new president and chief executive. Davis is best known for running the digital media company Corbis from 1993 to 2007, but, over the past five years, he has stepped into the global health arena, particularly though leadership positions at PATH, the Infectious Disease Research Institute and the Fred Hutchinson Cancer Research Center, all based in Seattle. "Steve understands about governance approaches in a different way, plus he's spent a lot of time in the field," says Lisa Cohen, executive director of the Washington Global Health Alliance. Davis replaces Chris Elias, who led PATH for 12 years and now serves as president of global development for the Bill & Melinda Gates Foundation.

### Cardio captain

After more than two-and-half years without a permanent director, the US National Heart, Lung and Blood Institute has tapped a new leader. Starting this summer, cardiologist Gary Gibbons (pictured), founder and director of the Cardiovascular Research Institute at the Morehouse School of Medicine in Atlanta, will replace acting director Susan Shurin at the helm of the $3.1-billion institute, the third largest among the NIH's 27 institutes and centers. Gibbons's research has focused primarily on the molecular and genetic bases of ethnic disparities in cardiovascular disease.

Morehouse School of Medicine

## RESEARCH

### Cancer compendiums

Tailoring cancer therapies to an individual tumor just got easier, thanks to two new databases that combine genetic testing with drug profiling of close to 1,000 diverse tumor cell lines. "This is going to enable us to realize the vision we have for personalized medicine," says Levi Garraway of the Dana-Farber Cancer Institute in Boston. Reporting in late March, Garraway and his colleagues described the Cancer Cell Line Encyclopedia, a collection of DNA and pharmacological information from 479 cell lines exposed to 24 anticancer molecules (*Nature* **483**, 603–307, 2012). Another team created a similar resource using 130 drugs and up to 507 cancer cell lines per agent (*Nature* **483**, 570–575, 2012). See go.nature.com/mFvFyZ for more.

### More online

**FoldIt game's next play:**
go.nature.com/wyr2qF

**Oversight for omics tests:**
go.nature.com/yxvDgu

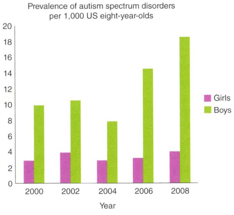
**Spinoff speculation spikes:**
go.nature.com/ZNDoNI

### Parasitic portent

Two studies published on 5 April indicate that malaria parasites on the Thailand-Myanmar border are rapidly developing resistance to the first-line treatment for the disease, artemisinin. An international team looked at medical records from around 3,200 people treated with artemisinin and found that the proportion of slow-clearing infections increased from 0.6% to 20% over the past decade (*Lancet* doi:10.1016/S0140-6736(12)60484-X, 2012). A companion paper published by the same group in *Science* (**336**, 79–82, 2012) identified a hotspot on chromosome 13 of the malaria parasite's genome that is associated with drug resistance in Southeast Asia. "It's critically important that we identify the genes responsible for resistance so we can conduct better surveillance and develop better treatments," says senior author Timothy Anderson, a geneticist at the Texas Biomedical Research Institute in San Antonio.

### More children 'on the spectrum' than ever before

Autism rates are at an all-time high in the US. According to the Centers for Disease Control and Prevention's (CDC's) biannual survey, approximately one in 88 children received a diagnosis of autism, Asperger's syndrome or a related disorder by age eight in 2008, with boys about five times more likely than girls to be labeled autistic (*MMWR Surveill. Summ.* **61**, 1–19, 2012). The latest prevalence figures, released on 29 March, reflect a near doubling in the frequency of autism spectrum diagnoses since the CDC started keeping records eight years ago. The trend can partly be explained by wider screening and better diagnosis. But, according to Mark Roithmayr, president of Autism Speaks, a New York–based advocacy group, "a large portion of the increase—some 50%—remains unexplained."



# SOUND MEDICINE

Illustrations by Sydney Smith

Everyone from rock stars to nonagenarians experiences hearing loss, but no drugs have ever been approved specifically to prevent or treat this problem. Recently, a handful of drug companies have started to make some noise, with a number of experimental compounds now in human trials. **Elie Dolgin** sounds off on what could be a multibillion dollar market.

Sonja Wittermann woke up in her Munich apartment with a dull pressure in her left ear. She got out of bed, but "I was not sure if I was standing right," Wittermann recalls. It was 1 April 2009, but this was no April Fools' joke. "I recognized that there was something very wrong."

Wittermann rushed to seek medical attention at the nearby Ludwig Maximilians University Grosshadern Clinic, where she was diagnosed with idiopathic sudden sensorineural hearing loss, a mysterious one-sided deafness condition with no known cause that affects approximately one in 10,000 people per year. Wittermann, 30 years old at the time, had lost 30% of the hearing in her affected ear.

The standard of care for treating idiopathic hearing loss, if caught early enough, is a gradually diminishing course of corticosteroids. According to a large US clinical trial published last year, more than 75% of people who experience sudden deafness respond to steroids if treated within two weeks, and around 20% totally recover their hearing[1]. Physicians, however, don't really know why the steroids work.

"For us, [steroid therapy] feels a lot like chicken soup—it won't hurt, it might help," says Steven Rauch, an otologist at the Massachusetts Eye and Ear Infirmary (MEEI) in Boston who led the work. "That's a simplification. It can hurt. But we're so comfortable with the spectrum of side effects that we feel pretty safe offering steroids as a treatment."

Because steroids are far from perfect, researchers and drugmakers have been on the hunt for more targeted drug therapies. One of these experimental agents was offered to Wittermann on that April day three years ago. Instead of receiving steroids, Wittermann, who helps run a large restaurant with a beer garden in Munich, signed up to participate in a phase 2 trial testing a compound known as AM-111. This peptide drug inhibits an enzyme called c-Jun N-terminal kinase, which is involved in so-called 'cell suicide', or apoptosis, in the inner ear, a major contributor to permanent hearing loss. She received an injection through her eardrum—an experience that sounded "like thunder," Wittermann says—and a week later half of the lost hearing returned. It has stayed that way ever since.

Wittermann still doesn't know if she received a placebo injection or one with the active drug, and the results of the trial, which launched in late 2008 and involved more than 200 people at some 20 sites across Germany, Poland and the Czech Republic, won't be known until later this year. But Markus Suckfüll, an audiologist at the Martha-Maria Hospital in Munich who is leading the trial, is cautiously optimistic. "Having an apoptosis inhibitor that you can use locally—I think that's a clever idea," he says. "The risks are very low, but the possible benefits are high."

AM-111, which is being developed by

the Swiss company Auris Medical, is one of only a few so-called 'otoprotective' drugs—compounds that stop damage in the ear before it starts or becomes permanent—in human testing. To date, "no large pharma company has a hearing-loss prevention branch," notes Kathleen Campbell, an audiologist at the Southern Illinois University School of Medicine in Springfield. "None."

That may soon change. Recently, the big players in the drug industry have started investing in the hearing space, mostly through partnerships and licensing agreements that remain in the preclinical stage. In 2010, for example, the Swiss drug giant Novartis inked a $214 million deal to co-develop gene therapy treatments for hearing loss together with Maryland-based GenVec. And, last year, France's Sanofi signed a two-year agreement with the Dutch biotech firm Audion Therapeutics to advance small-molecule drugs capable of regenerating sensory hair cells in the inner ear.

### Listening to the market

These actions speak to the growing recognition of a huge untapped market, with one in five Americans over the age of 12 now reporting some degree of hearing loss, according to a recent report[2]. Age-related hearing loss is the most common problem, notes Ralph Holme, head of biomedical research at Action on Hearing Loss, a London-based nonprofit. But "trying to design a clinical trial to test something against age-related hearing loss could be quite a headache," he says. The disease has many driving factors, and it typically progresses quite slowly. As such, "things like noise-induced hearing loss and ototoxicity will be more attractive."

They could also be quite lucrative: Action on Hearing Loss has pegged the drug market for noise-induced hearing loss at around $2 billion, with other hearing-related indications adding even more value. That's only a fraction of today's $6 billion hearing implants and devices market, but the new drugs could offer some key advantages. For one thing, drugs are neither uncomfortable nor cosmetically off-putting, two common complaints of hearing aids. But, moreover, otoprotective agents should offer real therapeutic value.

"Hearing aids and cochlear implants work well, but neither of them is like true hearing," says Jonathan Kil, president and chief executive of Sound Pharmaceuticals, a Seattle-based start-up with an experimental drug for hearing loss in phase 2 testing. "The ability to hear pure tones and to discriminate speech requires this complex effort, involving input from many structures in the cochlea, and that complexity demands some repair of critical structures and regeneration."

Still, despite ample preclinical evidence showing that many drugs work in various rodent models of noise-induced hearing loss, including mice, guinea pigs and chinchillas, translating those findings to humans has proven difficult. For one thing, notes David Weber, president and chief executive of San Diego–based Otonomy, finding participants for placebo-controlled trials is a tall order. "Both from a patient perspective and a clinical perspective, to be treating someone with a placebo when all the clinical evidence says there's a response to [existing] steroids is just hard to consider," Weber says. Last year, Otonomy reported positive phase 1 trial results showing that OTO-104, the company's novel sustained-release formulation of the steroid dexamethasone, improved the symptoms of an inner-ear disorder called Ménière's disease better than a placebo comparator. But, to complete that 44-person trial, the company had to enlist 15 hospitals across the US, and some of these sites, such as the MEEI, didn't manage to sign up a single participant.

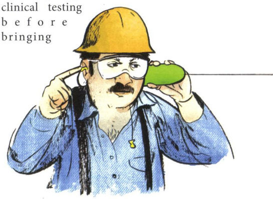
Trial recruitment is further complicated because participants need to be enrolled within the small timeframes during which the otoprotective drugs are expected to have therapeutic benefit. For example, in Auris's phase 1 study of the AM-111 compound that Wittermann later received, the company advertised on radio stations and in the subway systems of Munich and Berlin in late December 2005 in hopes of finding people who ultimately experienced firecracker-induced hearing loss during celebrations on New Year's Eve. "We probably had the world's shortest enrollment period," says company chairman and managing director Thomas Meyer. "It was just one single day: January 1st."

Yet, among all the residents of these two of Germany's most populous cities, only 28 individuals showed up on New Year's Day. Of these, the 11 who had experienced sufficient hearing loss to be eligible for the trial were treated between 11:40 a.m. and 10:20 p.m. (One person had a piece of a firecracker in his ear canal, but after the object was removed, his hearing improved and he didn't qualify for treatment anymore.) The phase 1 data looked promising, with two participants experiencing improvements of more than 28 decibels, a level that far exceeds what is normally seen in people who spontaneously recover from noise trauma[3]. But, on the basis of the recruitment experience in that small trial, Auris decided to advance the drug mostly for idiopathic, rather than noise-induced, hearing loss in its next phase of development.

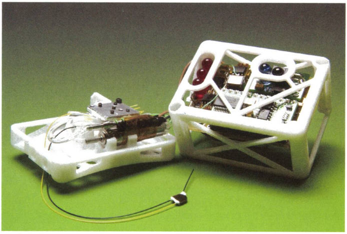
### Take two and hear me in the morning

Because AM-111 is injected into the middle ear, it must be administered by a trained health professional. From a practical standpoint, however, a pill that can protect hearing is preferable, especially for the many types of people who are exposed to continuous loud noises at the workplace, such as military personnel and factory workers. "Local administration to the cochlea is not a viable option for these people," says Eric Bielefeld, a hearing researcher at Ohio State University in Columbus. "Most noise-prevention drugs would need to be something you can take on a daily or semidaily basis." (Some researchers are even testing implants to deliver drugs directly to the cochlea of the inner ear, although those devices remain many years away from human testing; see 'Innermost desires'.)

One of the first modern oral drugs advertised specifically for hearing loss hit the market in 2003. 'The Hearing Pill' was sold online by San Diego's American BioHealth Group (ABG) as a 'nutraceutical', a type of dietary supplement that doesn't require clinical testing before bringing



## Innermost desires



Draper Laboratory

The inner ear is called the 'bony labyrinth'—and for good reason. This essential part of our hearing organ consists of a maze of skeletal cavities, including the snail-shell–shaped cochlea, the target of most agents designed to combat hearing loss. Unfortunately, getting drugs into the cochlea has proven a complex puzzle.

Many compounds in pills don't cross the blood-cochlear barrier and come with off-target effects, whereas drugs injected into the space behind the ear drum don't always permeate from the middle ear into the organ's inner recesses. Thus, for deeper drug penetration, some researchers have been working on implantable devices that can directly deliver compounds into the inner cochlear fluid.

The most advanced such model is being developed by Jeffrey Borenstein and his colleagues at the Charles Stark Draper Laboratory in Cambridge, Massachusetts. By combining a microfluidic pump, a drug reservoir, an electronic control system and tiny tubing, Borenstein's team has devised an apparatus that could one day be implanted in the temporal bone just behind the human ear to provide long-term drug delivery in a controlled and timed manner.

"Bringing it to clinical fruition is really what we're aiming to do," says Borenstein, whose group is working to miniaturize the device, with plans to build a prototype suitable for human clinical trials in 2015. "You can take out quite a bit of that [bone] and not do anything structurally that would cause any problems. That's what the surgeons tell me."

In collaboration with auditory scientists at the nearby Massachusetts Eye and Ear Infirmary in Boston, Borenstein's team is first testing a prototype on guinea pigs: a contraption about the size of a ring box, with all the components connected to a thin tube surgically implanted in the cochlea. The researchers had tried strapping the device (pictured) to the rodents' backs, but that didn't work. "We started referring to them as 'Little Houdinis' because they literally managed to get out of every jacket or backpack we designed for them," says Erin Pararas, a Draper scientist. The group eventually found a workaround by fastening the pods surgically to the top of the guinea pigs' heads.

Currently, the researchers are using the device to infuse a chemical called 6,7-dinitroquinoxaline-2,3-dione (DNQX), which temporarily blocks signals between sensory hair cells and nerve fibers leading to the brainstem by blocking glutamate receptors. Thanks to the fact that hair cells close to the entrance of the cochlea pick up higher-frequency sounds than cells further down the length of the coiled tube do, the researchers can track where the drug has traveled using a series of hearing tests. In the future, they plan to switch over to agents that protect or even help regenerate, rather than hinder, the ear's most delicate structure.

"Implantable-type devices are all in the fairly early stages," says David Borkholder, a biomedical engineer at the Rochester Institute of Technology in upstate New York who is working on an intracochlear drug delivery system in mice. "But I view them as an essential tool that will enable advanced deafness therapy research and development beyond what people do today." —*ED*

to market. Based on intellectual property developed by Richard Kopke, a former ear surgeon at the Naval Medical Center in San Diego, the drug contained an antioxidant called *N*-acetylcysteine (NAC), which is used as a prescription drug to counteract acetaminophen overdose and as a nutraceutical for its liver- and lung-protective functions.

In extensive preclinical testing, Kopke and many others showed that NAC protected against various forms of hearing loss, including loud noise, by scavenging the free radicals that form during and after the metabolic stress and contribute to acquired deafness. But the results from human testing were more lackluster. A 2003 Navy trial, the largest conducted to date, enlisted 566 Marine recruits undergoing two weeks of weapons training with M-16 rifles. Subjects in the still-unpublished study were randomized to receive 900 milligrams of NAC or placebo three times a day with each meal for the duration of the boot camp.

According to Kopke, who helped run the trial, less than a third of the recruits, all of whom wore ear plugs for protection, experienced any hearing loss, and, for those people who did, the NAC treatment only reduced the occurrence of significant hearing loss by about 25%. However, a similar follow-up study in another population of recruits-in-training that tested a higher dose of NAC and controlled for smoking and painkiller use found no such effect.

"There may be some suggestion of benefit, but there are some statistical measures that don't support this," says Michael Hoffer, a Naval Medical Center neurotologist who was not involved in the two trials but who has worked with the study authors testing NAC in other settings, including in soldiers fighting in Iraq.

### Beta testing

"The Hearing Pill was really a beta test for the concept, and the concept was, 'Will people buy an orally dosed nutraceutical to address and treat sensorineural hearing loss?'" says David Karlman, chief executive of American BioHealth Group. The answer was a resounding 'yes'. "We sold several thousand bottles."

Nonetheless, ABG stopped promoting the product to focus on a new combination approach that, according to Karlman, "will allow us to take the best of NAC and make it better." In 2009, Karlman, together with Kopke and Robert Floyd, head of experimental therapeutics at the Oklahoma Medical Research Foundation in Oklahoma City, launched Otologic Pharmaceutics to advance a dual-compound pill containing NAC and another antioxidant, α-phenyl-tert-butyl nitrone (HPN-07). In the 1990s, Floyd showed that HPN-07 helped protect against oxidative damage in the central nervous system, which then led the British drug giant AstraZeneca to develop the drug for the treatment of stroke. (It ultimately failed in phase 3 testing.)

Earlier this year, Kopke, Floyd and their colleagues reported that the two-drug combo, called NHPN-1010, significantly reduced the amount of damage to the ear's outer hair cells as well the synapses of the auditory nerve and the brainstem in rats exposed to blast-induced hearing loss[4]. "Our preliminary data looks like it's synergistic in its action. We find that HPN-07 seems to be more protective of the outer hair cells, and NAC might preserve neurons better," says Kopke, now retired from the Navy and working as chief executive of the Hough Ear Institute in Oklahoma City. "This is going to be quite a bit more effective than either drug by itself." In March, Otologic received $2.4 million from the US Department of Defense to launch a phase 1 trial of the combination in healthy individuals.

Even if NAC—with or without another drug—never fully pans out, many researchers are still hoping that other antioxidants will prove their therapeutic worth. For example, Kathleen Campbell is leading a 600-person, Department of Defense–funded trial testing oral liquid suspensions of an antioxidant amino acid called D-methionine given to drill sergeant instructors during and after their 11-day weapons training. Campbell, together with Colleen Le Prell of the University of Florida in Gainesville, is also planning to test a combination of antioxidant vitamins and minerals at either a military airbase or at a metal stamping factory in Spain.

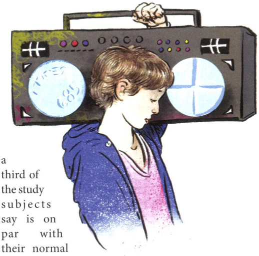
But Le Prell is not putting all of her eggs in one basket. "We're going to need more than one solution," she says. "We don't have one birth control pill, one cholesterol drug, one blood pressure medication. If we can develop many [hearing] drug options, everyone wins, especially the people who need it the most."

Last year, Le Prell teamed up with Seattle's Sound Pharmaceuticals to test SPI-1005, an oral preparation of a small molecule called ebselen that mimics and prompts production of the enzyme glutathione peroxidase 1. In animal models, ebselen has been shown to help stop oxidative injury and stress in a range of ailments, including diabetes, ischemic stroke, acoustic trauma and hearing loss caused by chemotherapies, such as cisplatin, which are well known to damage people's hearing ability. The drug was rejected by Japanese authorities after completion of clinical trials of the drug there for the treatment of stroke, owing to poor efficacy data. But Sound Pharmaceuticals' Kil remains confident that ebselen will help repair the hair cells of the outer ear and protect other cochlear structures in the inner ear, thereby reducing the levels of both temporary and permanent hearing loss.

A 32-person study completed in 2007 showed that SPI-1005 was safe and bioavailable in healthy people[5]. To follow up, Sound Pharmaceuticals had planned to conduct a phase 2 trial in military recruits undergoing weapons training at the US Naval Medical Center in San Diego (as Kopke had done with NAC). But, after failing to get that trial off the ground, the company turned to a new study population exposed to loud sounds: college students listening to music on their iPods. Earlier this year, Sound Pharmaceuticals launched an 80-person interventional trial led by Le Prell.

### Hear me baby one more time

The students in the iPod study are being given SPI-1005 or a placebo twice daily for four days and are then asked to listen to four hours of music through headphones. Study subjects can choose whether to listen to a selection of Top 40 pop songs, including hits by Britney Spears, Kelly Clarkson and Rihanna, or a rock music playlist with tunes by Aerosmith, Billy Idol and Bon Jovi, among others. Both options maintain a steady 100 decibels throughout—about the same loudness as a motorcycle—which around a third of the study subjects say is on par with their normal listening volume. They are then tested for any changes in their hearing thresholds. At the same time, the company is running an equally sized trial testing SPI-1005 for the treatment of chemotherapy-induced hearing loss at the US Department of Veteran Affairs Puget Sound Health Care System in Seattle.



Recently, even big pharma has started to enter the clinic. In December of last year, New York–based Pfizer started recruiting people with age-related hearing loss for a phase 1 trial involving a compound that is also in development for the treatment of schizophrenia.

Hearing experts say they are encouraged by the growing number of experimental treatments entering human testing, but, given the lack of phase 2 efficacy data, most are reserving judgment. "Until the final word is in, I think it's better to be hopeful than not, especially when dealing with medical treatments that can prevent hearing loss or recover hearing loss before it becomes permanent," says Gordon Hughes, program director for extramural clinical research at the US National Institute on Deafness and Other Communication Disorders in Bethesda, Maryland. "But we need more rigorous scientific evidence."

Given the heightened commercial activity in the hearing field, that plea does not seem to be falling on deaf ears.

***Elie Dolgin is a news editor with* Nature Medicine *in Cambridge, Massachusetts.***

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# The WHO must reform for its own health

Tikki Pang & Laurie Garrett

**The World Health Organization (WHO) is facing an unprecedented crisis that threatens its position as the premier international health agency. To ensure its leading role, it must rethink its internal governance and revamp its financing mechanisms.**

Tikki Pang/Viorel Florescu

The World Health Organization was born in the bifurcated Cold War world in 1948, and every aspect of its charter, mission and organizational structure was molded by diplomatic tensions between NATO and the USSR. However, with the collapse of the Soviet Union and the rise of the new emerging market superpowers, the WHO finds itself trying to straddle a global dynamic for which it was not designed.

Indeed, the WHO now finds itself marginalized in a crowded global health landscape characterized by poor coordination among multiple players. It is no longer the only major actor. At the same time, it faces an internal crisis, with major budget shortfalls and staff layoffs that have resulted in the organization embarking on the most radical reforms in its 64-year history[1]. But the changes do not go far enough. A recent dialogue on WHO reform that we participated in, held by the Council on Foreign Relations in New York in February, identified several key challenges that should be addressed by the agency.

First and foremost, the WHO should refocus on its original aim of being primarily a 'knowledge broker' that gives advice and information about best practices but stops short of directly implementing programs[2]. It should convene negotiations resulting in internationally binding legal agreements and monitor their implementation. Some of its most successful achievements—such as the Framework Convention on Tobacco Control, the International Health Regulations and the International Classification of Diseases—fall into this category.

The means by which such agreements are reached has changed, and the organization needs to adapt. In 1948, the WHO acted as a knowledge-and-standards broker between states, working almost exclusively with ministries of health and government leaders. In the twenty-first century, however, the WHO's credibility and relevance depend on its ability to exert a normative influence through the Internet, informing the global citizenry about all aspects of health—from relevant treaties to drug safety to disease outbreaks. Currently, the organization's website, http://www.who.int, is nearly impossible to navigate, akin to a well-stocked library with no catalog system. It needs an overhaul to be useful to the global citizenry.

The WHO not only needs to better communicate and coordinate with its global partners; it also needs to make improvements within, starting with its internal governance. The organization must enhance the relationship between its Geneva headquarters and its powerful regional offices. Guidance from Geneva is sometimes ignored, even contradicted, by the regional directors and their offices. Although the WHO was born with a clear top-down leadership structure, it has morphed over the decades into something closer to a partnership: Geneva 'suggests' policies that its regional partners may accept, ignore or amend. It is often difficult to tell whether the tail is wagging the dog. For example, the Pan American Health Organization, which is one of the regional offices of the WHO, may choose to design and implement a Chagas disease eradication strategy having sought little or no input from Geneva. To avoid tensions, the organization should more clearly apportion 'core' versus 'support' roles played by the various parties[3].

The internal changes must also involve improved finances. In 1990, the agency was by far the largest player on the global health field, with an annual budget of nearly $1.2 billion; the next biggest budget at the time was that of US government global health programs, which totaled $850 million. By 2010, the WHO's budget, after years of increases, fell back to that 1990 level, making it the fourth largest spender in the global health landscape, behind the now-mammoth $7.5 billion US program, the $3 billion Global Fund to Fight AIDS, Tuberculosis and Malaria and the $2.2 billion collective pile of smaller nongovernmental organizations. This year, the WHO seems to be falling further behind in the hierarchy, trailing the GAVI Alliance and the Bill & Melinda Gates Foundation.

Until recently, the WHO garnered more than 80% of its budget in the form of voluntary donations, largely given by the wealthiest countries for earmarked programs. The agency's core support is derived from proportional levies on member nations, which have remained unchanged for years despite the rising costs of WHO operations. Moreover, the WHO's revenues are received in US dollars, but its Geneva operational and payroll costs must be met in Swiss francs. Because the WHO has not practiced currency hedging, a 32% increase in the value of the franc against the dollar, as occurred in 2011, cannot be accommodated without severe institutional fiscal pain.

In addition to practicing currency hedging, the WHO must identify a range of financing innovations with a goal of increasing institutional resilience. Such financing mechanisms may include, for example, the establishment of an endowment fund, a multiyear financing framework, or the use of a Robin Hood tax, which reaps financing from miniscule taxation of very large currency transactions. Both of these options were highlighted by a 5 April report from a consultative expert working group convened by the WHO. And, like any multibillion-dollar company, the WHO should have an effective 'marketing' strategy built around rigorous, external evaluations that demonstrate the value of its activities.

The world needs an aggressive and scientifically solid health leader. Governance and the setting of normative standards cannot be accomplished with a slew of loosely connected health initiatives, nongovernmental organizations and bilateral programs. The only entity with a charter, a legislative body and a mandate to fill that role is the WHO, and it must do so decisively.

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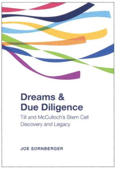
# BOOK REVIEW

## Founding a new field



**Dreams & Due Diligence: Till and McCulloch's Stem Cell Discovery and Legacy**

**Joe Sornberger**

University of Toronto Press, 2011
184 pp., hardcover, $29.95
ISBN: 1442644850

### Reviewed by David Nathan

Joe Sornberger is a professional writer commissioned by the Canadian Stem Cell Foundation to create a monograph on the Canadian legacy of the collaborative efforts of James Till and the late Ernest 'Bun' McCulloch, whose seminal work was the discovery of a mouse bone marrow cell that they called the spleen colony-forming cell.

In a study designed to determine radiation sensitivity of mouse bone marrow cells, they noticed unexpected lumps on the spleens of the irradiated and bone marrow transplanted mice. Several of those lumps turned out to be colonies of blood cells derived from progenitors capable of giving rise to granulocytes, megakaryocytes or erythrocytes.

A splendidly written foreword by Alan Bernstein, a leader of Canadian biomedical science and a former student of the researchers, puts their work in clear scientific perspective. Their discovery, as well as the vital contributions of Donald Metcalf and his co-workers in Australia and of Leo Sachs in Israel, both of whom developed culture conditions that permitted much simpler growth of hematopoietic colonies *in vitro*, set the stage for a subsequent explosion of information on the regulation of normal and malignant hematopoiesis.

The book is based on a series of interviews with Till and McCulloch and with many of their most distinguished students at the Ontario Cancer Institute (aka the Princess Margaret Hospital) in Toronto whose independent careers have brought substantial credit to Canadian biomedical science.

Sornberger profitably explores the relationship between the two men. Unlike many successful scientific collaborators, such as Nobel Prize winners Michael Brown and Joseph Goldstein, who discovered the pathways that regulate serum cholesterol, or Michael Bishop and Harold Varmus, who demonstrated the role of oncogenes and their derivation from viruses, the approaches of Till and McCulloch to scientific work were totally orthogonal. They also came from widely disparate social and scientific backgrounds. But friendships are often built because each of the partners sees in the other qualities that they desire in themselves. Till and McCulloch made a landmark discovery largely because they were capable of observing and appreciating the importance of completely unexpected findings. The ability to notice and pursue the unanticipated result is key to both excellent science and clinical care.

Sornberger is a writer, not a critical scientist, so readers will not gain much insight into the history of the study of hematopoieisis before and after Till's and McCulloch's seminal finding. And there is a bit of Canadiocentric chauvinism expressed in the pages. For example, Ronald Worton, former Scientific Director of the Ottawa Health Research Institute, is a splendid geneticist and a major contributor to inherited muscular diseases, but he is not *the* discoverer of the muscular dystrophy–associated gene, as Sornberger seems to suggest. It is strange that he does not mention that Louis Kunkel of Children's Hospital in Boston and his colleagues made key contributions to that discovery. Tak Mak of Toronto is an outstanding molecular biologist, but not *the* discoverer of the T cell receptor. But this criticism might be considered caviling. The fact is that the students of Till and McCulloch are highly distinguished members of the scientific, and particularly hematologic, communities.

In the final section of the book, Sornberger launches into a discussion of the controversy over human embryonic stem cells. However, it is a very long stretch from the classic experiments of Till and McCulloch, who focused largely on the relatively mature myeloid stem cells that produce granulocytes, platelets and red blood cells, to the totipotential embryonic stem cells that are capable of giving rise to all of the cells of the body. Nonetheless, the author plunges into the furiously debated morass of embryonic stem cell research without adding very much resolution to the conflict. He is properly outraged that unscrupulous hucksters are selling injections of so-called 'stem cells' to desperate patients, but hucksterism has been part of the US if not the Canadian medical culture since colonial days. That it persists is a terrible disgrace.

Toward the book's end, the author decries the fact that Till and McCulloch never received a Nobel Prize. But that is probably not due to lack of appreciation of their work. In my opinion, this failure relates to the difficulty that the Nobel Committee must have had when they tried to limit the assignment of the prize to three people. The development of the field depended on both *in vivo* (Till and McCulloch) and *in vitro* (Donald Metcalf and Leo Sachs) experiments, and four researchers are simply one too many for the Nobel Prize. That particular prize notwithstanding, theirs was truly a seminal discovery.

In summary, this is an interesting short book because the author interviewed some of the best experimental hematologists in the world to gather the data he needed to write it. Their thoughts about the field and about Till and McCulloch are valuable archives. The writing style is clear, but the material is not groundbreaking. It stands as a tribute to a collaboration between two totally disparate individuals who came together to do a great experiment and expanded a great cancer center, the Ontario Cancer Institute. That may be reason enough to catch the attention of basic and physician scientists.

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**COMPETING FINANCIAL INTERESTS**

The author declares no competing financial interests.

# Activation of BK channels may not be required for bitter tastant–induced bronchodilation

**To the Editor:**

Deshpande *et al.*[1] reported that bitter tastants increase intracellular $Ca^{2+}$ concentration (to similar levels produced by the bronchoconstrictive agonists histamine and bradykinin) yet cause marked bronchodilation. This implies that elevated $Ca^{2+}$ concentration inhibits contraction, challenging the classic $Ca^{2+}$-dependent mechanism underlying smooth muscle contraction[2,3]. To resolve this apparent paradox, the authors showed that bitter tastants can generate localized $Ca^{2+}$ events, and that bitter tastant-induced relaxation and hyperpolarization can be inhibited by the large-conductance $Ca^{2+}$-activated $K^+$ (BK) channel antagonist iberiotoxin[1]; thus, they propose that bitter tastant–induced bronchodilation results from its ability to generate localized $Ca^{2+}$ signals, which in turn open BK channels and hyperpolarize the membrane. However, their assertion of the involvement of BK channel activation was solely based on the effect of iberiotoxin on bitter tastant–induced relaxation and change in membrane potential as assessed by voltage-sensitive dyes. We are concerned that the association of BK channel activity with relaxation has not been directly examined, raising questions about the proposed relaxation mechanism. We therefore directly studied the effect of bitter tastants on the activity of BK channels and examined the relaxation effect of multiple BK channel inhibitors in mouse airway smooth muscle, the same type of smooth muscle tissue used in Deshpande *et al.*[1].

To directly investigate the effect of bitter tastants on BK channel function, we used patch-clamp technology (see **Supplementary Methods**), an unequivocal methodology for studying ion channel activity. We first examined the effects of the bitter tastant chloroquine on spontaneous transient outward currents (STOCs). These currents result from the opening of BK channels in response to local, short-lived $Ca^{2+}$ events, that is, $Ca^{2+}$ sparks, thus representing reliable readouts of the BK channel activity in these cells[4,5]. If bitter tastants generate localized $Ca^{2+}$ events as Deshpande *et al.*[1] have suggested, it is plausible that they would increase the activity of STOCs. However, we found that chloroquine at 1 mM, a dose that causes total relaxation (Fig. 3c in Deshpande *et al.*[1]), did not increase STOC amplitude or frequency within the first minute or so of application (**Fig. 1a,b**; $n = 9$). To our surprise, about 2 min after application chloroquine began to inhibit and then completely block STOCs (**Fig. 1a,b**). The inhibition was reversible (**Fig. 1a**), indicating that chloroquine at this concentration does not cause appreciable damage to the cells.

BK channels are gated by both $Ca^{2+}$ and membrane potential, and hence we investigated the effect of chloroquine on depolarization-evoked $K^+$ currents. Upon depolarization from –70 mV to –15 mV, mouse airway smooth muscle cells produced a peak current of 104 ± 8 pA (**Fig. 1c**; $n = 14$). Chloroquine (1 mM) inhibited this current by 35% 2 min after application (**Fig. 1c**; $P < 0.05$ with paired $t$ test, $n = 8$). Similarly, iberiotoxin (100 nM) suppressed this current by 30% (data not shown; $P < 0.05$ with paired $t$ test, $n = 6$).

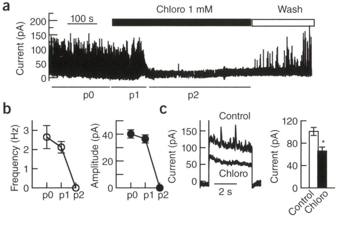

**Figure 1** Effect of chloroquine on BK channel currents in mouse airway smooth muscle cells. The cells were isolated and voltage-clamped as described in the **Supplementary Methods**. The reversal potential for $K^+$ was set at –82 mv and for $Cl^-$ at –15 mv. (**a**,**b**) A representative long recording of the effect of chloroquine (Chloro) (1 mM) on STOCs at a holding potential of –15 mV (**a**). Control (p0), phase 1 (p1) and phase 2 (p2) are marked beneath the trace and correspond to the time periods used to calculate the mean values of STOC frequency and amplitude shown in **b**. Data are means ± s.e.m., $n = 9$. (**c**) Two representative recordings of voltage-evoked $K^+$ currents in the presence of 1 mM chloroquine and in its absence (left) and currents (means ± s.e.m.) as measured at 200 ms after the onset of depolarization ($n = 8$; $*P < 0.05$) (right). The currents were activated by depolarization to –15 mV from a holding potential –70 mV. In the control, STOCs were evoked in addition to the sustained $K^+$ current. The experimental protocols for mouse research were approved by the Institutional Animal Care and Use Committees at the University of Massachusetts Medical School.

These results prompted us to reexamine bitter tastant–induced relaxation and the effect of BK channel blockers on this relaxation in isolated mouse airway. The bitter tastants quinine, chloroquine and denatonium all relaxed methacholine-induced contraction and did so in a concentration-dependent manner (**Fig. 2a,b**), consistent with the results of Deshpande *et al.*[1]. At 1 mM, within 4.5 ± 0.4 min ($n = 29$), chloroquine relaxed methacholine-induced contraction by 91.3 ± 2.4%. The extent of this relaxation in intrapulmonary mainstem bronchi (**Fig. 2b,c**) was comparable to that in trachea and extrapulmonary mainstem bronchi (**Supplementary Fig. 1a,b**). However, chloroquine (1 mM) still fully reversed methacholine-induced contraction in the presence of 100 nM iberiotoxin (**Fig. 2d**). This is in contrast to the result of Deshpande *et al.*[1], who showed that chloroquine only partially reverses the contraction under the same conditions. The reasons for this discrepancy are not known. However, because of this discrepancy, we also examined the effect of iberiotoxin at 300 nM. We found that at this high concentration iberotoxin still exerted no effect on chloroquine-induced relaxation (**Fig. 2d,e**). These results indicate that bitter tastants do not activate BK chan-

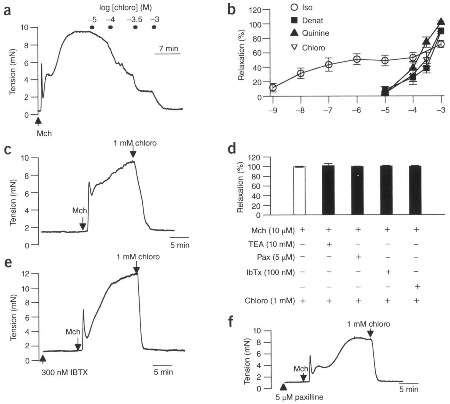

**Figure 2** Lack of an effect of BK channel blockers on bitter tastant–induced relaxation in mouse intrapulmonary mainstem bronchi. (**a**) A representative recording of chloroquine (Chloro)-induced, concentration-dependent relaxation response in a bronchus precontracted with 100 μM methacholine (Mch). (**b**) Concentration responses of relaxation caused by denatonium (Denat), quinine and chloroquine. As a comparison, the concentration-response to a β agonist, isoproterenol (Iso), is also included. To construct these curves, cumulative concentrations of bitter tastants and isoproterenol were administered to the bronchi precontracted with 100 μM methacholine. (**c**–**f**) BK channel blockers failed to change chloroquine-induced relaxation in the bronchi precontracted with 10 μM methacholine. Traces are representative recordings of chloroquine-induced relaxation in the control (**c**) and in the presence of 300 nM iberiotoxin (IbTx) (**e**) or 5 μM paxilline (**f**); the bar charts (**d**) depict the mean values at conditions indicated at the bottom. BK channel inhibitors were added to the bath 16–20 min before Mch stimulation. For both **b** and **d**, data are means ± s.e.m. of five to eight individual experiments; $^*P < 0.05$. TEA, tetraethylammonium. The experimental protocols for mouse research were approved by the Animal Care and Use Committee of the Model Animal Research Center of Nanjing University, China.

nels to cause the relaxation. To further confirm this, we examined the effects of paxilline and tetraethylammonium on bitter tastant-mediated bronchodilation. Paxilline at the micromolar level and tetraethylammonium at the low-millimolar level are known to block BK channels in airway smooth muscle[4,6]. Paxilline at 5 μM and tetraethylammonium at 10 mM failed to prevent relaxation caused by bitter tastants in both airways from trachea and mainstem bronchi (**Fig. 2d,f** and **Supplementary Fig. 1b**). Finally, in the airways starting at 5 mN tension, the level used in the study of Deshpande *et al.*[1], paxilline and tetraethylammonium failed to inhibit bitter tastant–induced relaxation (**Supplementary Fig. 1c,d**).

Our results confirm the finding of Deshpande *et al.*[1] that bitter tastants produce strong bronchodilation in airways precontracted by methacholine. But direct recording of BK channel current with patch clamp reveals that bitter tastants do not activate BK channels. Moreover, three different BK channel inhibitors were unable to alter the relaxation effect of bitter tastants. Therefore, it is unlikely that the activation of BK channels contributes to bitter taste receptor–mediated bronchodilation as Deshpande *et al.*[1] asserted. Notably, we found that bitter tastants inhibit and block BK currents in the time window when they cause bronchodilation. It remains to be determined whether this BK current inhibition is linked to the bronchodilation induced by bitter tastants. Blocking BK channels could inhibit membrane hyperpolarization and decrease the driving force for nonselective cation channels presumably activated by methacholine stimulation[7], or this effect could be a mere epiphenomenon. Our results demonstrate that bitter tastants could activate an unknown relaxation mechanism in airway smooth muscle, and further experiments are needed to uncover this mechanism.

Our results also suggest the necessity to further explore other aspects of the mechanism proposed by Deshpande *et al.*[1]. Although Deshpande *et al.*[1] reported that bitter tastants alone increase global $Ca^{2+}$ concentration, it remains unknown how they affect this $Ca^{2+}$ concentration in the presence of methacholine. It is well established that methacholine increases global $Ca^{2+}$ concentration in airway smooth muscle[8,9]. So it would be useful to test whether bitter tastants suppress methacholine-induced increases in global $Ca^{2+}$ concentration, resulting in bronchodilation. Moreover, Deshpande *et al.*[1] used only cultured airway smooth muscle cells to study bitter tastant–induced global and local $Ca^{2+}$ signals[1]; it would be desirable to determine whether similar $Ca^{2+}$ signals can be detected in native airway smooth muscle cells. A clear understanding of the above $Ca^{2+}$ signals, and possibly other relaxation processes, should shed light on the mechanism underlying bitter tastant–induced bronchodilation. This would facilitate translating this class of promising potent relaxants to the bedside for patients with asthma.

*Note: Supplementary information is available on the Nature Medicine website.*

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**An *et al.* reply:**

Zhang *et al.*[1] responded to our paper[2] in which we showed that bitter taste receptors (TAS2Rs) mediate relaxation of airway smooth muscle by increasing the concentration of intracellular calcium ($[Ca^{2+}]_i$). We are pleased to see that they also found TAS2R agonists to markedly relax airways, with similar efficacies to those that we found[2]. In our study we showed that TAS2R function in airways was mediated by $[Ca^{2+}]_i$ release on the basis of the concordance of relaxation potency and efficacy with measured $[Ca^{2+}]_i$ release, the loss of TAS2R-mediated relaxation when $[Ca^{2+}]_i$ release was blocked and the stimulation of spatially and temporally distinct $[Ca^{2+}]_i$ events evoked by TAS2R agonists in intact airway smooth muscle. We considered that large-conductance $Ca^{2+}$-activated $K^+$ channels ($BK_{Ca}$) were activated, and thus played some part in the relaxation response, on the basis of the sensitivity of TAS2R-mediated relaxation of mouse airways and individual airway smooth muscle cells to the highly specific $BK_{Ca}$-channel inhibitor iberiotoxin (IbTx). However, we showed that IbTx only partially blocked relaxation (Fig. 3b,c of the paper[2]) to bitter tastant, leaving open the possibility of additional mechanisms.

To further address the concerns of Zhang *et al.*[1], we first examined whether blockade or direct activation of $BK_{Ca}$ by IbTx or NS1619, respectively, results in the expected changes in airway smooth muscle tone. In mouse trachea studied *ex vivo*, 100 nM IbTx caused a 15% ± 5.3% increase in force over baseline ($P = 0.01$, $n = 14$), whereas 100 μM NS1619 caused a 91% ± 6.0% decrease in force of methacholine-contracted airways ($P < 0.01$, $n = 4$). These results indicate that $BK_{Ca}$ activation status is capable of regulating the airway smooth muscle contractile state in a dynamic fashion and in a direction consistent with receptor-mediated channel opening resulting in bronchodilation. Next, we reexamined the effects of IbTx on methacholine-contracted mouse airways using the TAS2R agonist chloroquine, the same agent used by Zhang *et al.*[1]. Mouse (FVB/N strain) tracheas in the *ex vivo* model were studied with full dose-response studies to ascertain potential nonspecific effects of chloroquine.

Consistent with our original report[2], IbTx partially blocked the relaxation effect of this TAS2R agonist in mouse trachea, with an ~35% decrease in efficacy (**Fig. 1a**). This effect is not ambiguous and is highly reproducible. It is unclear why Zhang *et al.*[1] find no effect of IbTx, but potential differences due to mouse strains[3,4] or methodology might explain this discrepancy. We used 100 μM methacholine versus the 10 μM concentration used by Zhang *et al.*[1] to contract the rings, and we performed studies starting with low concentrations of chloroquine and increasing them in a dose-response fashion (as opposed to a single high dose), which might have a priming effect. Finally, it has been established that mouse airway physiological and $[Ca^{2+}]_i$ responses are highly dependent on the anatomical location of the segment[5]. Studies of human airways can be helpful to assure the applicability of findings in mice. And, indeed, there are many known differences in receptor responsiveness to G protein–coupled receptor agonists between mouse and human lung[6,7]. In our original paper[2] (as well as in this reply), we used human airway smooth muscle cells or airways whenever possible because of this potential issue. We have now studied IbTx sensitivity of bitter tastant responses in rings from fourth- and fifth-order human bronchi. We found between-sample variation in relaxation and the extent of the IbTx effect, with the most apparent IbTx effect being noted at 500 μM doses of chloroquine (**Fig. 1b**). The variability may be due to underlying pathology, exposure history or differing anatomical regions of the airway, but overall these findings demonstrate that blockade of $BK_{Ca}$ with IbTx results in physiologically relevant loss of TAS2R-mediated relaxation in human airways (**Fig. 1b**).

We performed additional studies with isolated airway smooth muscle cells, again derived from human samples, examining mechanics in the absence of any influence from the epithelium. We used Fourier transform traction microscopy[8,9] to measure spatiotemporal changes in the contractile stress generated by an individual human airway smooth muscle cell. **Figure 1c** shows representative traction fields before and after exposure to chloroquine, in the absence or presence of IbTx. The cell boundary is shown by the white line, and colors show the magnitude of the tractions in Pascals (Pa). In most of these intracellular areas, traction was markedly reduced by chloroquine (**Fig. 1c**, top). Furthermore, IbTx treatment blocked some, but not all, areas of the chloroquine-mediated traction responses (**Fig. 1c**, bottom), indicative of partial blockade of the relaxation effect of the TAS2R agonist in human airway smooth muscle. We performed additional quantitative studies using magnetic twisting cytometry. In five independent experiments representing >1,500 airway smooth muscle cell measurements (**Fig. 1d**), we found that chloroquine relaxed human airway smooth muscle cells to a maximum of 34% ± 0.8% ($n = 717$) at 90 s after exposure (the 150 s point of **Fig. 1d**), whereas in the presence of IbTx relaxation at that same time point was 14% ± 1.6% ($n = 808$, $P < 0.001$). As we previously described[2], there is an initial relaxation response to chloroquine that is not sensitive to the toxin (**Fig. 1d**), and this may represent an additional component that is manifested in the coordinated relaxation of the intact airway that is IbTx insensitive.

We previously used voltage-sensitive dyes to ascertain changes in membrane potential[2] on the basis of reports of correlations with other forms of whole-cell measurements, including patch-clamp technology[10,11]. We agree, though, that voltage-sensitive dyes do not represent direct measurements and may be less useful for dissecting complex pathways, particularly when new and paradoxical signaling events seem to be at play.

Given the physiologic responses that we observed in four different models, we find that blockade of $BK_{Ca}$ substantially impairs, but does not ablate, TAS2R-mediated airway relaxation. We conclude that $BK_{Ca}$ activation by localized $Ca^{2+}$ events promoted by TAS2R agonists is one part of a complex mechanism by which these receptors relax airway smooth muscle and that this mechanism is required for the full physiological effect of receptor activation. Additional studies will be required to ascertain other partners in this multifactorial end-organ response. Nevertheless, TAS2Rs remain attractive targets as direct bronchodilators for the treatment of obstructive airway disease. Finally, we propose that, whenever possible, studies in this area should be performed in human rather than mouse airway smooth muscle cells and airways, as the receptor and effector responses of airways are differentially manifested (often markedly so[6,7]) between

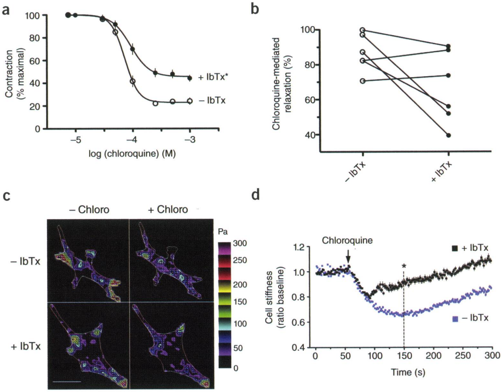

**Figure 1** Airway smooth muscle responses to the TAS2R agonist chloroquine are sensitive to the $BK_{Ca}$ antagonist IbTx. (**a**) Mouse airway relaxation in response to chloroquine in the absence (control) or presence of 100 nM IbTx. Results are from nine independent experiments. $*P < 0.05$ versus control. (**b**) Human airway relaxation due to 500 μM chloroquine in the absence (control) or presence of 100 nM IbTx. Shown are the individual results from six rings derived from two subjects. Data are scaled to the maximal response in the group. For experiments in **a** and **b**, airway rings were studied in a lateral isometric myograph, with baseline passive tension set at 5 mN, and then contracted with 100 μM methacholine. (**c**) Representative traction maps of single human airway smooth muscle cells determined by Fourier transform traction microscopy. The colors represent the magnitude of the regional tractions in Pa indexed to the color bar at the right. Results are representative of four experiments. Scale bar, 40 μm. (**d**) Quantitative measurements of cell relaxation ascertained by magnetic twisting cytometry. Results are from individual measurements from 717 cells exposed to 1.0 mM chloroquine alone (–IbTx) and 808 cells pretreated for 30 min with 100 nM IbTx followed by exposure to 1.0 mM chloroquine. Data are from five independent experiments and are shown as mean ± s.e.m. $*P < 0.001$ versus –IbTx at 90 s after chloroquine exposure (the 150 s point of the *y* axis). These studies were approved by the Institutional Animal Care and Use Committee of the University of Maryland and the Institutional Review Board of Johns Hopkins University. Tissues in **b**–**d** were from discarded surgical specimens.

the species. Additionally, it is well recognized that, between human and mouse bitter taste receptor families, there are multiple expressed receptors without genetic or pharmacologic homologs[12].

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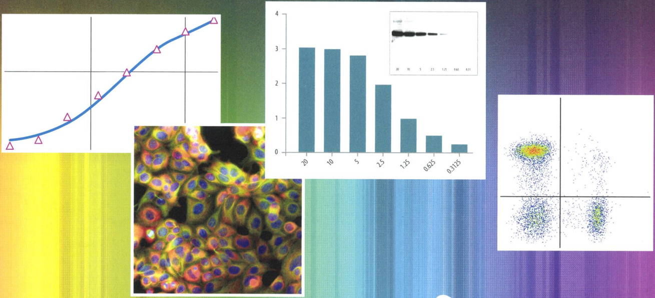

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# Mitochondria to the rescue

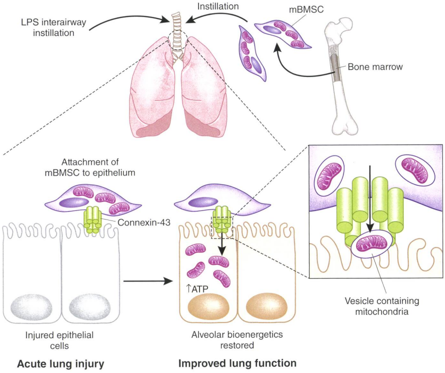
Darwin J Prockop

**A new study using a mouse model of lung diseases is the first demonstration *in vivo* that bone marrow–derived stromal cells can repair tissue injury through the transfer of mitochondria (pages 759–765). This suggests that rescue of injured cells through mitochondrial transfer may be an important process in many diseases.**

Much like the initial response of classical physicists to the discovery of subatomic particles, most biologists are skeptical about cell-to-cell communication that occurs through vesicles or particles—it's perhaps too messy and confusing. Nevertheless, there is conclusive evidence that cells can communicate through the transfer of vesicles and particles carrying lipids, proteins, mRNAs, miRNAs and DNAs[1–7].

Shedding of vesicles or particles by cells has been reported extensively over the last 50 years. One of the earliest reports was the observation that human plasma contained particulate 'dust', subsequently defined as vesicles, from activated blood platelets[1]. Other early reports showed increased amounts of vesicles or particles in synovial fluid or blood from patients with arthritis and other inflammatory diseases[2]. Still other reports demonstrated that cancer cells, virally infected cells, vascular cells and injured tissues shed cellular components[3–7]. Moreover, some of the shed components contained mRNAs and miRNAs that transferred genetic information to the target cells[6,7]. In this issue of *Nature Medicine*, Islam *et al.*[8] report that the transfer of an organelle as large as a mitochondrion can also occur and can contribute to the repair of an injured lung *in vivo*, observations that have broad implications for the process of tissue repair.

Islam *et al.*[8] explored the molecular basis of a previous report showing that lethal lung injury induced with bacterial lipopolysaccharide (LPS) in mice was rescued by administration of the stem/progenitor cells from bone marrow referred to as mesenchymal stem or multipotent stromal cells (BMSCs)[10]. The authors used a technology that they previously developed for real-time microscopy of isolated and perfused lungs from mice. The data demonstrated that BMSCs infused into the trachea of the mice attached to LPS-injured alveolar epithelial cells by forming connexin-43–containing gap junctions. They further showed that, after the attachment, labeled mitochondria were transferred from the BMSCs to the LPS-injured alveolar cells, apparently through the formation of nanotubes and microvesicles[8]. The transfer of mitochondria was accompanied by a restoration of ATP levels in the alveolar cells and expansion-induced secretion of surfactant by these cells. In addition, control experiments showed that BMSCs did not transfer mitochondria and did not improve survival of the LPS-injured mice in the absence of wild-type connexin-43 or functional mitochondria.


LPS interairway instillation
Instillation
mBMSC
Bone marrow
Attachment of mBMSC to epithelium
Connexin-43
↑ATP
Vesicle containing mitochondria
Injured epithelial cells
Alveolar bioenergetics restored
Acute lung injury
Improved lung function

Marina Corral

**Figure 1** Mitochondrial transfer from BMSCs protects against lung injury. Islam *et al.*[8] used a mouse model for pulmonary disease created by infusing a bacterial extract of LPS into the trachea of mice. BMSCs, which were also similarly infused into the mice, attached to alveolar epithelial cells through connexin-43–containing gap junctions. After the attachment, mitochondria were shown to transfer from the BMSCs to the LPS-injured alveolar cells, a process that seems to be mediated through nanotubes and microvesicles. This transfer of mitochondria was accompanied by restored ATP levels in the alveolar cells, promoting tissue repair and increasing survival of mice. mBMSC, mouse BMSC.

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Transfers of cellular components occur through structures that are highly heterogeneous, and they have been seen in multiple contexts. As a result, there has been a profusion of names to describe them. The most common names are microvesicles or microparticles (MVs/MPs), terms used to include fragments of plasma membrane, and specialized extracellular organelles, such as nanotubes and filopodia[5]. But cell-to-cell communication through MVs/MPs poses enormous difficulties in defining regulation and control of the process. In effect, the observations require a conceptual leap from the seductive paradigm that the fates of cells are primarily controlled by ligand-receptor interactions on the plasma membrane that are transmitted by signaling pathways to the nucleus.

Until recently, transfer of a mitochondrion seemed beyond the capacity of MVs/MPs. Mitochondria were first shown to be transferred between cells though filopodia-like extensions in co-cultures of BMSCs and a line of lung epithelial cells with nonfunctioning mitochondria (A549 $\rho^0$ cells)[11]. Two subsequent publications—where human BMSCs were co-cultured with rat cardiomyocytes[12] or with human osteosarcoma cells depleted of functional mitochondria[13]—confirmed this cell-to-cell transfer phenomenon, but only *in vitro*. One of the first indications that mitochondrial transfer might occur *in vivo* came from the unusual observation that the host mitochondrial DNA was found in a venereal tumor transmitted in feral dogs[14]. The report by Islam *et al.*[8], however, is the first demonstration that transfer of intact mitochondria can contribute to tissue repair *in vivo* (**Fig. 1**).

One consequence of the evidence of mitochondrial transfer is that the phenomenon probably explains some of the beneficial effects that have been observed with administration of BMSCs in animal models for a large number of diseases[10]. These BMSCs were originally thought to repair tissues by engrafting and differentiating to replace injured cells; however, beneficial effects were commonly observed without long-term engraftment of the cells. Instead, they seem to repair tissues, or limit damage from excessive inflammatory and immune responses, by being activated by signals from the injured tissues to secrete a large number of paracrine factors. Given that BMSCs remain in most tissues for only a short time, the sequence was referred to as 'kiss and run'. Islam *et al.*[8] suggest a 'Trojan horse' effect in transferring mitochondria, even though they point out that their results do not exclude the possibility that paracrine factors secreted by the BMSCs may have contributed to the tissue repair they observed.

The transfer of functional mitochondria to injured cells can itself have profound effects, as any decrease in reduction of oxygen by mitochondria generates highly toxic reactive oxygen species. The reactive oxygen species, in turn, activate inflammasomes and other components that trigger the normal cascade of inflammatory and immune responses. Many diseases are now recognized to involve tissue destruction from excessive or unresolved inflammation[10]. Therefore, transfers of functional mitochondria to cardiomyocytes during a myocardial infarction or to neurons during a stroke could interrupt a deadly progression of events. And perhaps therapies with mitochondria, instead of cells, might be possible for some diseases. In one series of experiments[10], adding isolated mitochondria to cultures of mitochondria-defective cells had no effect; however, the possibility was not explored in detail.

The observations on mitochondrial transfers may have much broader implications. To date, it has only been seen from BMSCs, cells that are found in the adventitia of most blood vessels and that are mobilized by tissue injury[10]. It seems likely that some of the many other types of cells that participate in tissue repair can also serve as donors of mitochondria. Moreover, in three reports[8,10,13], the transfer has only been seen if the target cells had nonfunctional mitochondria or were exposed to the toxic effects of LPS. Therefore, it seems likely that transfer of mitochondria from several kinds of donor cells to injured cells may be a key component of tissue repair in many pathological conditions.

COMPETING FINANCIAL INTERESTS
The author declares competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemedicine/.

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# Germs gone wild

Namiko Hoshi & Ruslan Medzhitov

**The normally harmless behavior of bacteria in the intestinal tract is maintained by community structure and the integrity of host defenses. When either or both of these are compromised, a few disgruntled outcasts can cause a riot, taking down the whole neighborhood (pages 799–806).**

Trillions of bacteria colonize the lower intestinal tract in mammalian species. The composition, diversity and stability of the intestinal microbial ecosystems have recently become a subject of intense investigation and a rich source of surprising findings. One notion that quickly emerged from these studies is that loss of microbial diversity in colonized tissues tends to be associated with deleterious effects on the host[1]. Oral administration of antibiotics has one of the most powerful impacts on microbial composition and diversity and has long been known to disrupt the intestinal homeostasis, predisposing the host to infections with antibiotic-resistant pathogens[2]. In this issue of *Nature Medicine*, Ayers *et al.*[3] provide a detailed account of the lethal consequences of antibiotic-induced disturbance of the intestinal microbiota and the detrimental role of the innate immune system in decreasing tolerance in this setting.

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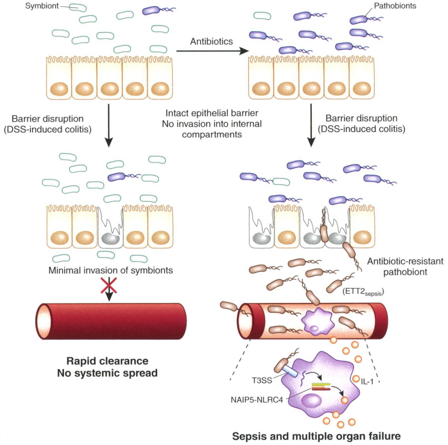
Symbiont
Pathobionts
Antibiotics
Barrier disruption (DSS-induced colitis)
Intact epithelial barrier
No invasion into internal compartments
Barrier disruption (DSS-induced colitis)
Minimal invasion of symbionts
Antibiotic-resistant pathobiont
($ETT2_{sepsis}$)
Rapid clearance
No systemic spread
T3SS
IL-1
NAIP5-NLRC4
Sepsis and multiple organ failure

Marina Corral

**Figure 1** Epithelial integrity and microbial composition affect intestinal homeostasis and the innate immune response. Pathobionts are normally present at low density in a microbial community dominated by symbionts (top left). Ayres *et al.*[3] show that antibiotic-mediated depletion of symbionts leads to expansion of antibiotic-resistant pathobionts; however, an intact epithelial barrier prevents their invasion (top right). Disruption of epithelial integrity (as occurs after DSS-induced colitis) can lead to a transient invasion of symbionts, but these are rapidly cleared by the immune system (bottom left). In the context of antibiotic-induced dysbiosis, however, disruption of the epithelial barrier leads to invasion of pathobionts and subsequent septicemia. *E. coli* O21:H+ carries a variant of a virulence gene cluster known as $ETT2_{sepsis}$, which may promote flagellin secretion through the T3SS. Flagellin secreted into host macrophages activates the NAIP5–NLRC4–IL-1β pathway, ultimately leading to multiple organ damage caused by the systemic inflammatory effects of IL-1β.

Studies in germ-free animals have long established a crucial role of microbial colonization in the gut for normal development and physiology of the host organism[4]. Administration of broad-spectrum antibiotics to adult mice bypasses the developmental effects of colonization and can recapitulate some of the phenotypes of germ-free mice, such as alteration of intestinal epithelial homeostasis and increased susceptibility to damage[5]. However, antibiotics do not completely sterilize the intestinal lumen, and antibiotic-resistant bacterial strains can greatly expand in the newly available niches. The consequences of such an expansion depend on the host status—for example, barrier integrity and immune competence—as well as on the identity of the antibiotic-resistant bacteria.

The study by Ayers *et al.*[3] shows how antibiotic treatment affects intestinal epithelial damage caused by dextran sodium sulfate (DSS). DSS is toxic to colonic epithelium, and its oral administration is a convenient and widely used model of intestinal damage and colitis. Antibiotic-induced depletion of commensal bacteria was previously shown to increase susceptibility to DSS-induced intestinal injury[5]. In that model, commensal bacteria provided ligands for Toll-like receptor (TLR) stimulation, which, in turn, was necessary to promote epithelial tissue protection and repair. In the experimental model used by Ayers *et al.*[3], antibiotic administration after DSS treatment resulted in severe mortality, but the cause of the problem seems to be a disruption of normal intestinal bacterial composition by antibiotics rather than a lack of stimulation.

Depletion of resident bacteria (symbionts) that normally dominate the intestinal niches resulted in the overgrowth of an antibiotic-resistant strain of pathogenic *Escherichia coli* (*E. coli* O21:H+) (**Fig. 1**). Interestingly, the virulence gene cluster of *E. coli* O21:H+ has a characteristic feature shared with other septicemic *E. coli*[3]. Indeed, invasion of *E. coli* O21:H+ into the systemic circulation caused a sepsis-like condition with severe inflammatory tissue damage in multiple organs[3]. This invasion was facilitated by DSS-mediated disruption of intestinal barrier integrity. However, intravenous injection of the bacteria into mice untreated with DSS was sufficient to cause sepsis and mortality, confirming the connection between this particular pathogen and the disease.

Notably, the lethal systemic inflammatory response triggered by *E. coli* O21:H+ was dependent on the production of interleukin-1 (IL-1β) through the activation of NAIP5-NLRC4 inflammasome in macrophages. Inflammasomes are intracellular protein complexes that control proteolytic processing and secretion of IL-1β and several other inflammatory mediators[6]. NAIP5-NLRC4 recognizes bacterial flagellin[7], which can be secreted into the host cell through the specialized secretory apparatus known as the ETT2 type 3 secretion system (T3SS)[8,9]. The *E. coli* O21:H+ isolated by Ayers *et al.*[3] and related septicemic bacteria share a genetic variation (known as $ETT2_{sepsis}$) affecting their T3SS, suggesting that $ETT2_{sepsis}$ may be particularly prone to flagellin secretion. It would be interesting to determine in future studies whether all bacteria expressing this particular version of the T3SS can cause sepsis through the flagellin–NLRC4–IL-1β pathway. This could inform the choice of therapy (such as IL-1 blockade) for patients with sepsis, as sepsis can be caused by more than one mechanism and in some settings TNF-α has a more dominant role compared to IL-1β[10].

Immunopathology, such as collateral tissue damage caused by the immune response, is often an unavoidable cost of host defense against infections. The clinical challenge of dealing with immunopathology is that reducing the immune response can compromise host protection from an ongoing infection. How to strike the right balance between optimal host protection and minimal immunopathology is not always clear, and it requires a detailed knowledge of the relative contribution of different host defense mechanisms to protection versus disease. In that context, another key finding by the authors is that the NLRC4–IL-1β pathway does not seem to contribute to the clearance of the pathogenic *E. coli* O21:H+, as blocking this pathway did not affect pathogen burden, even though it did improve host survival[3]. This indicates that in this model of sepsis, interfering with the NAIP5–NLRC4–IL-1 β pathway might be beneficial without having negative consequences for host immunity. Furthermore, it can be therapeutic even in the face of high bacterial burden, which can be particularly relevant for antibiotic-resistant pathogens such as *E. coli* O21:H+. Indeed, when infections progress to sepsis, the inflammatory response is a far greater evil than the direct tissue damage by the pathogen—at that stage, suppressing pathogen load alone has a limited therapeutic value. Therefore, identifying the pathways responsible for the inflammatory tissue damage but dispensable for host immunity, as done by Ayers *et al.*[3], is of utmost importance.

Finally, the study by Ayers *et al.*[3] highlights another key emerging issue: the notion of pathobionts, microorganisms that can be part of the normal microbiota in healthy hosts but that can cause disease when some aspect of host-microbial homeostasis is disrupted[11]. The difference between pathobionts and opportunistic pathogens is that the latter are traditionally thought to be able to cause the disease when the host immunity is compromised. The definition of pathobionts can be considerably broader, as their pathogenic potential can be unleashed by multiple factors, particularly alterations of microbial ecology in the colonized host[12]. Perhaps the most famous microorganism exemplifying this principle is *Helicobacter pylori*—although more than half of humans harbor this bacterium in their stomachs, only a fraction of them develop ulcers[13]. The distinctions between beneficial commensal microbes, pathobionts and pathogens are not absolute, yet the emerging evidence suggests that, in a given host, the presence and relative abundance of pathobionts seem to determine the susceptibility to multiple inflammatory diseases[14,15]. The 'germ theory' of disease proposed that many human maladies can be caused by specific pathogens. In the near future, we may expect an expansion of the germ theory to incorporate the pathogenic potential of normal constituents of our microbiota.

COMPETING FINANCIAL INTERESTS
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# Metabolic surgery for type 2 diabetes

David E Cummings

**Clinicians note that bariatric operations can dramatically resolve type 2 diabetes, often before and out of proportion to postoperative weight loss. Now two randomized controlled trials formally show superior results from surgical compared with medical diabetes care, including among only mildly obese patients. The concept of 'metabolic surgery' to treat diabetes has taken a big step forward.**

To combat the twin pandemics of obesity and type 2 diabetes mellitus (T2DM), clinicians need every tool they can get. Major, durable weight loss is uncommon with medical and/or behavioral approaches, and adequate glycemic control often remains elusive. Many diabetes drugs promote weight gain, and using them to normalize blood glucose increases the risks of hypoglycemia. The most effective intervention for weight loss and T2DM seems to be bariatric surgery. Because some operations engage weight-independent antidiabetes mechanisms[1,2], using them to treat T2DM in less-obese patients is under consideration[3,4]. The full risk/benefit ratio of surgical versus nonsurgical approaches, however, has not been adequately tested. US National Institutes of Health (NIH) guidelines based on body mass index (BMI) criteria for patient selection have governed bariatric surgery for over 20 years[5]. Yet these recommendations were crafted with neither any Level 1 evidence addressing surgical versus nonsurgical methodologies nor an emphasis on T2DM. Now two randomized controlled trials (RCTs) comparing bariatric operations with medical therapy to treat T2DM, including among patients below the usual BMI threshold for surgery, report that surgery yields better glycemic control, diabetes remission and reduction of other cardiovascular risk factors, with seemingly acceptable complications, for 1–2 years[6,7].

NIH guidelines from 1991 limit bariatric surgery to people with a BMI $>40$ kg m$^{-2}$, or $>35$ kg m$^{-2}$ plus obesity-related complications[5]. Although these recommendations were generated without relevant RCTs comparing surgical with nonsurgical care, two decades of clinical observations seem at face value to validate them for severely obese patients. Among such patients, nonrandomized studies report that bariatric surgery is associated with reductions in cardiovascular risk factors[8], cardiovascular events[9] and mortality[10,11]. In contrast, medical or lifestyle interventions for obesity and diabetes have never been proven to reduce cardiovascular events or death.

Despite these compelling clinical observations, RCTs of surgery versus nonsurgery are sorely needed. Ample precedents exist wherein RCTs reversed longstanding paradigms derived from nonrandomized clinical trials[12,13]. Some of the best evidence in bariatric surgery, from the Swedish Obese Subjects study (a long-term observation of various operations versus conventional care), is prone to allocation bias because participants were not randomized[8–10]. Subjects who actively chose surgery may be more motivated overall and generally take better care of themselves. The NIH is unlikely to reconsider its guidelines without pertinent RCTs, and insurance

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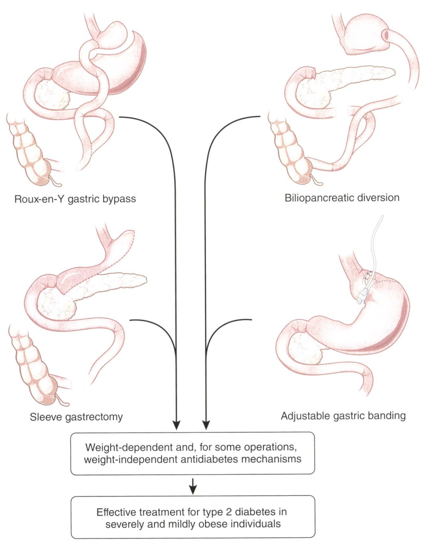
Roux-en-Y gastric bypass
Biliopancreatic diversion
Sleeve gastrectomy
Adjustable gastric banding
Weight-dependent and, for some operations, weight-independent antidiabetes mechanisms
Effective treatment for type 2 diabetes in severely and mildly obese individuals

Marina Corral

**Figure 1** Different methods of bariatric surgery ameliorate type 2 diabetes to varying degrees. Despite the differences regarding surgical method and levels of diabetes remission achieved, surgical procedures seem to promote more beneficial glycemic effects than medical therapy or lifestyle changes alone, including among people who are only mildly obese. New guidelines should be considered for recommending these surgical procedures on the basis of glucose homeostasis and metabolic disease rather than just on body weight.

companies are unlikely to pay for operations that are not NIH-sanctioned.

NIH guidelines also pay little attention to T2DM, yet bariatric operations, especially intestinal-bypass variants, exert powerful antidiabetes effects[1,2]. Short-to-moderate-term diabetes remission rates are 80–85% after Roux-en-Y gastric bypass (RYGB) and even higher after biliopancreatic diversion (BPD) (**Fig. 1**)[14]. These remarkable benefits result in part from mechanisms beyond reduced food intake and body weight. Hence, it seems logical to consider surgery to treat diabetes primarily, even in less-obese patients. Experts at the Diabetes Surgery Summit[2,3] and International Diabetes Federation[4] recommended surgery for T2DM in selected individuals with a BMI as low as 30 kg m$^{-2}$, but these recommendations were crafted with scant RCT data.

Although randomizing patients into interventions as diverse as surgery or medical care is extremely difficult, the above-mentioned trials provide crucial data regarding 'diabetes surgery'. Mingrone *et al.*[6] examined RYGB and BPD, whereas Schauer *et al.*[7] studied RYGB and sleeve gastrectomy, all of which were compared with medical and lifestyle therapy for T2DM (**Fig. 1**). Both studies showed better results after surgery for diabetes remission, improved glycemic control and reductions in other cardiovascular risk factors, including dyslipidemia and hypertension. In the first trial, postsurgical diabetes remission rates were dramatic—75% after RYGB, 95% after BPD and 0% with medical therapy alone[6]. Results seemed less striking in the second study—42%, 37% and 12% diabetes control after RYGB, sleeve gastrectomy and medicines, respectively[7]. This between-trial heterogeneity probably arises from differences in the primary endpoint, which in Mingrone *et al.*[6] was a glycated hemoglobin value <6.5% in patients off all diabetes medications, as opposed to ≤6.0% (with or without medications) in Schauer *et al.*[7] Although fewer patients achieved the latter study's endpoint, glycated hemoglobin reduction after surgery was remarkable—from ~9.4 to ~6.5%—with greatly decreased diabetes medication usage.

These two studies have several complementary features. One was relatively small but had longer follow-up (2 years) and compared conventional medical care; the other was larger but had only 1 year of follow-up and compared a more intensive medical and lifestyle program. Mingrone *et al.*[6] studied severely obese patients (BMI ≥35 kg m$^{-2}$), validating existing NIH guidelines with Level 1 evidence. Schauer *et al.* included subjects with a BMI as low as 27 kg m$^{-2}$, showing that surgical benefits extend to T2DM-affected individuals who are mildly obese or merely overweight; notably, no one suffered excessive weight loss or malnutrition. Although surgery proved superior for ameliorating diabetes in both studies, neither trial implemented a rigorous, supervised diet and exercise intervention, such as Look AHEAD[15]. It remains to be seen how this would fare when compared directly with surgery. The two studies complement previous RCT data showing greater T2DM remission after adjustable gastric banding (**Fig. 1**) compared with medical care[16]. Those participants had very mild diabetes, however, whereas the current studies[6,7] showed favorable surgical results in individuals with far more severe, longstanding disease.

Although these trials were not designed primarily to compare one operation with another, preliminary contrasts are possible. Compared with RYGB, BPD yielded better glycemic control (despite equivalent weight loss) at the expense of greater surgical complications. BPD also improved lipids better than did RYGB, except for high-density lipoprotein, which increased impressively only after RYGB. Compared with sleeve gastrectomy, RYGB tended to promote superior weight loss and glycemic control, but differences were modest. As sleeve gastrectomy creates relatively minor gastric restriction, without intestinal bypasses, the mechanisms mediating its impressive effects remain largely opaque.

As expected, all operations promoted greater weight loss than did nonsurgical interventions, but weight-independent mechanisms of diabetes remission were suggested for RYGB and BPD.

In Mingrone *et al.*[6], there was no correlation after either operation between the magnitude of changes in body weight and glucose levels, and neither baseline weight nor postoperative weight loss predicted diabetes remission or glucose lowering. Moreover, glycemic improvements preceded substantial weight loss. In Mingrone *et al.*, all surgical subjects achieved glycemic control without diabetes medications within only 15 days after surgery. In Schauer *et al.*[7], diabetes medication use plummeted postoperatively, long before maximum weight loss. Thus, the new studies corroborate a growing body of evidence showing that weight-independent mechanisms contribute to diabetes remission after some bariatric operations.

Numerous questions remain. What is the proper timing of surgery relative to other T2DM therapies? Because long diabetes duration is the strongest predictor of postoperative nonremission[2], surgery could be considered earlier in the disease, rather than as salvage therapy[4]. What are optimal criteria for patient selection? Although BMI is the standard, neither baseline weight nor postsurgical weight loss predict surgical benefits to glycemia[6] or cardiovascular disease[9]. Such benefits seem more related to improved glucose homeostasis than weight loss, and measures of the former (for example, fasting insulin levels) might constitute better surgical criteria than BMI[9].

How do various operations compare with one another in RCTs powered to see smaller differences? What is the full surgical risk/benefit ratio in less-obese patients with T2DM, and how low a BMI might we contemplate? Should we consider ethnicity because populations such as Asians develop T2DM at lower BMIs? What about long-term effects? Emerging data suggest that rates of diabetes recrudescence after initial postoperative remission can be high; however, even a transient disease-free period might confer long-lasting benefits from 'metabolic memory'. RCTs measuring important clinical endpoints, such as cardiovascular events, would be preferable to existing studies of surrogate endpoints like glycated hemoglobin. Such studies require thousands of participants. Given how difficult it is to randomize people into surgical versus nonsurgical interventions, novel methods for recruiting subjects in equipoise are needed, and relevant efforts are under way.

Nevertheless, bariatric surgery is a safe, effective treatment for T2DM, and is likely to benefit people with diabetes and only mild obesity. Several operations activate weight-independent antidiabetes mechanisms[1,2], rendering benefits related to improved glycemia more than weight loss[9], and BMI is a suboptimal stand-alone surgical criterion. Overall, it is time to view these operations as metabolic, not just bariatric, surgery.

COMPETING FINANCIAL INTERESTS

The author declares competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemedicine/.

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# Protective inflammasome activation in AMD

Jing Chen & Lois E H Smith

**Age-related macular degeneration (AMD) is the most common cause of blindness in the elderly. AMD progression is associated with alterations in inflammatory pathways and the immune system. A new study identifies a protective role for inflammasomes in AMD, suggesting that inflammasome activation might be manipulated as a potential therapeutic strategy for this condition (pages 791–798).**

Every year, millions of elderly adults worldwide lose their ability to read, drive or recognize faces as a result of age-related macular degeneration. Fine vision is lost in the central visual field (the macula), resulting in blurred images or a central 'black hole' (scotoma). Although people with AMD may have sufficient peripheral vision to allow them to perform basic daily activities, many ordinary tasks normally taken for granted, such as reading, can become challenging. As the number of aging people in the world increases, AMD will become an ever-increasing health burden in the next several decades. Thus, understanding the pathogenesis of AMD is crucial to finding ways to treat or prevent the vision loss associated with this disease.

AMD is a complex, multifactorial disease with both environmental and genetic risk factors, many of which are associated with inflammation and immunity. Both smoking and low omega-3 fatty-acid intake are strongly associated with AMD[1,2], and both are also associated with increased inflammation. Genetic studies have linked innate immunity (complement factor H) with AMD[3]. It is generally believed that increased inflammation contributes to AMD progression, and suppression of inflammation is needed for disease prevention.

The appearance of white or yellow lipid deposits beneath the retina, called drusen, marks early AMD. These deposits gradually separate the retinal pigment epithelium (RPE) from the underlying choroidal vascular bed, which provides oxygen and nutrients to photoreceptors. The RPE also helps clear cellular debris such as shed photoreceptor outer segments, which are renewed daily to replenish the enzymes necessary for light activation. The health of these photon-sensitive photoreceptor cells in the retina relies on a functional RPE-choroid interface. As drusen deposits increase in number, size or both, the proper functions of RPE and choroid are disrupted, and the photoreceptors degenerate, resulting in 'dry' or non-neovascular AMD. Abnormal leaky

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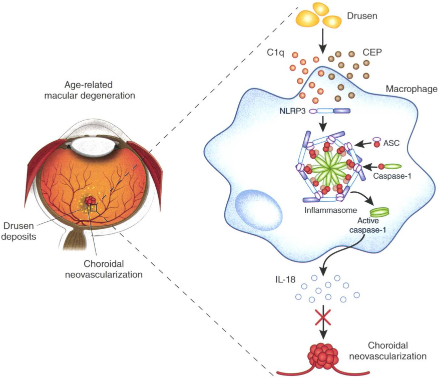
Age-related macular degeneration
Drusen deposits
Choroidal neovascularization
Drusen
C1q
CEP
Macrophage
NLRP3
ASC
Caspase-1
Inflammasome
Active caspase-1
IL-18
Choroidal neovascularization

**Figure 1** Inflammasome-mediated regulation of choroidal neovascularization in AMD. On the left, drusen deposits (yellow spots) and CNV are shown in the macula of the human eye. Doyle *et al.*[5] now provide evidence to show that drusen and their components C1q and CEP, once endocytosed by macrophages, can activate NLRP3 inflammasomes[5], in a complex with caspase-1 and ASC. Inflammasome activation may result in increased production of the inflammatory cytokine IL-18, leading to suppression of CNV.

blood vessels may also arise from the choroid growing through disrupted RPE into the subretinal space, causing rapid and substantial vision loss in 'wet' or neovascular AMD (**Fig. 1**). Determining the pathways that regulate progression from the often asymptomatic dry to the sight-threatening wet AMD is of prime importance. Previously, local chronic inflammation was suggested to have a key role in the formation of drusen on the basis of analyses of drusen components[4]. Activation of innate immunity (in particular the complement system) is generally believed to induce cellular damage in the retina, leading to choroidal neovascularization (CNV).

In this issue of *Nature Medicine*, Doyle *et al.*[5] provide evidence that some aspects of inflammation may be beneficial and slow the transition from dry to wet AMD through the activation of a protective inflammasome response. Inflammasomes are a group of recently discovered multiprotein complexes composed of an inflammatory component such as NOD-like receptor family, pyrin domain–containing 3 (NLRP3) and an apoptotic component (such as caspase-1), which are connected through an adaptor protein ASC (also known as PYCARD)[6] (**Fig. 1**). They are expressed in inflammatory cells, including macrophages, and can be activated by a diverse range of microbial or host damage signals to assemble into oligomers and initiate the apoptotic cascade[6].

Doyle *et al.*[5] found that NLRP3 inflammasomes can be activated by drusen isolated from eyes of donors with AMD and by the known drusen components C1q (a protein complex in complement activation) and carboxyethyl-pyrrole (CEP), an oxidative protein modification derived from docosahexaenoate-containing lipid[4]. Mice immunized with CEP had AMD-like lesions, drusen accumulation and other characteristics of dry AMD[7], and the authors found that NLRP3 and caspase-1 were induced in the macrophages near AMD-like lesions in these mice[5], suggesting that inflammasome activation occurs near lesion sites.

Upon their activation, NLRP3 inflammasomes can increase the production of inflammatory cytokines such as interleukin-1β (IL-1β) and IL-18 to mediate inflammation and tissue responses[8]. Using a laser-induced CNV mouse model of wet AMD, Doyle *et al.*[5] showed that lack of NLRP3 or IL-18 exacerbates CNV formation and that this is likely to be mediated through IL-18–regulated expression of the proangiogeneic factor vascular endothelial growth factor. Together these data indicate that, through NLRP3 and IL-18, activated inflammasomes have a protective role in AMD progression. It is not known, however, whether depletion of other inflammasome components (such as ASC and caspase-1) will similarly worsen CNV. It will also be interesting to see whether enhanced inflammasome activation or increased levels of IL-18 can suppress CNV. Nevertheless, the protective role of inflammasomes in AMD observed by Doyle *et al.*[5] challenges the existing dogma that global suppression of inflammatory processes will prevent AMD. This study suggests a paradigm-shifting concept: that controlled inflammatory responses may actually be beneficial in the initial onset of AMD (as in other situations such as infection). Only when the inflammatory response fails to repair tissue damage and becomes dysregulated does it have detrimental effects[9].

The newly revealed protective role of inflammasomes and their regulation of inflammatory cytokines in AMD also suggest new targets for control of AMD. Much of the current research on AMD pathogenesis has focused on chemokines, such as CCL2 and CX3CR1, that help recruit macrophages to the lesion sites[10–12]. Doyle *et al.*[5] direct attention to the pathways within macrophages that are activated to influence the progression from dry to wet AMD. Inflammasome-mediated processes are recognized as important steps in sensing both exogenous (for example, microbial) and endogenous (such as stress and tissue-derived) signals that not only guard against infection but also regulate autoimmune responses and multiple metabolic processes such as obesity and diabetes[6]. Now it seems that inflammasome activation also has a key role in sensing extracellular deposits and cellular damage in AMD to mediate disease progression through IL-18.

This study and future studies on the role of inflammasomes in AMD might lead to new treatments based on manipulation of inflammasome activation and promotion of the protective inflammatory response, as well as direct modulation of IL-18. Currently, there is no standard treatment for dry AMD except dietary supplementation with vitamins, antioxidants and dietary lipids that are associated with decreased risk of progression to wet AMD. For wet AMD, suppression of CNV with vascular endothelial growth factor–specific antibody therapy has been remarkably successful and is now the first line of treatment. However, preventive and noninvasive therapies for AMD would be preferable. Development of such therapies will largely depend on additional fundamental research into AMD to decipher the complex network of inflammatory and other mediators in this devastating blinding disease.

**COMPETING FINANCIAL INTERESTS**
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# King of hearts: a splicing factor rules cardiac proteins

Wolfgang A Linke & Sandra Bücker

**Alternative splicing ensures the expression of functionally diverse proteins from individual genes; however, aberrant mRNA splicing is associated with various conditions, including heart disease. A recent study provides new mechanistic insights into heart failure by showing that a human cardiomyopathy-linked mutation in a cardiac splice factor affects post-transcriptional regulation, causing the expression of anomalous isoforms of a whole network of cardiac proteins (pages 766–773).**

Heart muscle diseases, or cardiomyopathies, rank among the most prevalent causes of premature death in the Western world. A subset of cardiomyopathies is heritable, and causative mutations have been detected in almost 50 different genes so far. Among these primary cardiomyopathies, the most common form is dilated cardiomyopathy (DCM), which has been associated with more than 30 known disease genes. In DCM the heart becomes weakened and enlarged and cannot pump blood efficiently. The most frequently mutated gene in human DCM is *TTN*, which encodes the elastic muscle protein titin, the largest protein in the human body[1]. Notably, titin is also the focus of a new study published in this issue of *Nature Medicine* by Guo *et al.*[2], which provides new insights into the pathology of a hitherto underappreciated form of familial DCM. The authors concentrate on individuals with DCM in whom a component of the cardiac splicing machinery called RNA binding motif protein-20 (RBM20) is mutated[3]. The mutation of RBM20 leads to defects in splicing, triggering the expression of aberrant isoforms of titin, as well as of a network of other myocardial proteins that are collectively regulated by RBM20.

An important component of the study of Guo *et al.*[2] was the use of a rat strain with a spontaneously occurring mutation, which the same team had previously shown to result in the expression of irregular titin isoforms during heart development[4]. Mammals have a single gene that encodes titin, and the differences between titin isoforms largely arise from alternative splicing of the 'molecular spring region' of the protein (**Fig. 1**), which is a main determinant of myocardial passive (diastolic) stiffness. Normally, fetal hearts express a large N2BA-type titin isoform of approximately 3,700,000 Da[5]. This isoform is replaced around birth by smaller N2BA variants and the N2B isoform (~3,000,000 Da), which is the predominant titin isoform in the adult heart and is the least compliant of these titin isoforms. This perinatal titin isoform switch leads to the stiffening of the titin springs of adult cardiac sarcomeres. Strikingly, the team had previously shown that the hearts from rats homozygous for the spontaneously occurring mutation express a giant titin isoform called N2BA-G (3,830,000 Da) but no N2B isoform throughout life, making the adult cardiomyocytes from these mutant rats more compliant than normal[4]. Moreover, rats heterozygous for the mutation expressed large N2BA-type titins and low amounts of N2B, even in adulthood[4]. These pathological changes suggested anomalous alternative splicing of titin was occurring in the mutant rats.

Using genetic mapping, Guo *et al.*[2] have now identified the cause of the aberrant pattern of titin isoforms in the previously identified mutant rats. They showed that the rats have a missense mutation in the gene encoding the splicing regulator RBM20 that removes all of the gene except for the first exon. *RBM20* mutations have previously been detected in ~3% of human subjects with DCM[3,6], and Guo *et al.*[2] found yet another one (S635A). The authors used an RNA deep-sequencing approach to compare the cardiac tissue transcriptomes from subjects with DCM with and without *RBM20* mutations, as well as those from wild-type and *Rbm20*-mutant rats. They found that *RBM20* mutations dramatically altered not only titin isoform expression but also the splicing of 30 other cardiac proteins, most of which have been previously implicated in cardiomyopathy and heart failure. Importantly, the composition of this network of RBM20-regulated proteins was conserved between humans and rats. The authors largely excluded potential secondary effects on splicing as a result of heart disease.

It remains to be determined which RBM20 substrates are pivotal for the disease phenotype in DCM. Whereas people with an *RBM20* mutation often have clinically aggressive DCM[3,6], heterozygous RBM20-deficient rats show less severe, DCM-like symptoms, such as mildly enlarged hearts, fibrosis and a somewhat increased risk for arrhythmias and sudden cardiac death[2]. The subset of proteins aberrantly spliced in human *RBM20*- and rat *Rbm20*-mutant hearts includes molecules predicted to be involved in sarcomere structure and function, ion transport and $Ca^{2+}$ handling (**Fig. 1**). The functional impairment of any one protein from this network, or a combination thereof, could cause the DCM phenotype. For example, loss of function of titin alone could be responsible for DCM, as mutations in the titin-spring region can predispose to arrhythmogenic right ventricular cardiomyopathy[7]. Furthermore, the preferential expression of abnormally large titin isoforms in human *RBM20*-mutant hearts, as documented by Guo *et al.*[2], would be expected to make cardiomyocytes less stiff

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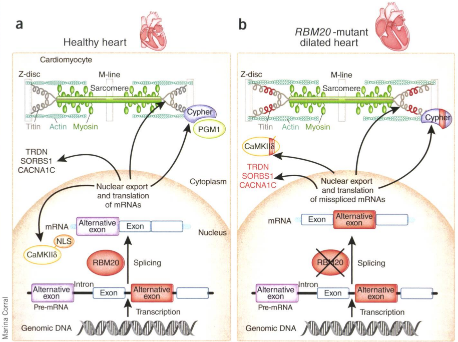

Marina Corral

**Figure 1** Loss-of-function mutations in the splicing factor RBM20 cause dilated cardiomyopathy via the pathological splicing of cardiac proteins. (**a**) Normally, DNA is transcribed into pre-mRNA, from which introns are removed and exons reconnected in multiple ways to generate mature mRNA. The mature mRNA is then exported from the nucleus and translated into protein. In a healthy heart, splice factors such as RBM20 are part of the machinery that controls mRNA exon splicing patterns. (**b**) Guo *et al.*[2] now show that loss-of-function mutations in RBM20 result in aberrant mRNA splicing patterns of cardiac proteins, for example, by the inclusion of a different alternative exon (shown in red). Misspliced mRNAs are exported from the nucleus, leading to the expression of pathological protein isoforms in cardiomyocytes. In rat *Rbm20*- or human *RBM20*-mutant hearts, the inclusion of extra exons in the sarcomere protein titin makes the spring region of this protein much larger and more extensible than usual. Guo *et al.*[2] also identify other splicing changes in cardiac proteins. In the protein kinase CaMKIIδ, exons 15 and 16 are included, but exon 14, encoding a nuclear localization signal (NLS), is removed, leading to mistargeting of the kinase to the sarcolemma of the cardiomyocytes. In the sarcomere-associated protein cypher, exons 5 and 6 are included, but exon 4, encoding a binding domain for the metabolic enzyme phosphoglucomutase-1 (PGM1), is removed, so that PGM1 can no longer bind cypher. Aberrant exon splicing patterns in *RBM20*-mutant hearts are also found for almost 30 other cardiac proteins, including triadin (TRDN), sorbin and SH3 domain–containing protein-1 (SORBS1) and the α1C subunit of the L-type calcium channel (CACNA1C).

and thus weaken the heart, resembling earlier findings in cases of idiopathic human DCM where titin was shown to become larger and more compliant[8]. It may be interesting for researchers to explore whether human *RBM20*-mutant hearts do indeed show impairments of calcium homeostasis, myocyte contractility or other cardiac cellular functions.

Like other splicing regulators, RBM20 is associated with the spliceosome, a ribonucleoprotein complex that removes introns from a transcribed precursor mRNA during the splicing process. More and more mutations in components of this splicing machinery are being shown to give rise to an increasing number of human diseases. Although cardiomyopathies are only beginning to be explored in this regard, recent evidence has suggested that RNA splicing is broadly altered in human heart failure[9]. RBM20 is expressed in substantial amounts only in striated muscle, most highly in the heart. This organ specificity is noteworthy, as tissue-specific alternative splicing is usually regulated by a combination of tissue-specific and ubiquitously expressed RNA-binding factors[10]. Whereas RBM20 is largely cardiac specific, two factors previously shown to regulate processing of mRNAs expressed in the heart, SC35 (ref. 11) and ASF/SF2 (ref. 12), are ubiquitously expressed. Both SC35 and ASF/SF2 are arginine/serine-rich (RS) proteins, which are known essential splicing regulators, and RBM20 is also an RS-related factor. SC35, ASF/SF2 and RBM20 show redundancy in terms of some of their splicing substrates[2,11,12]. However, important differences also exist: the splicing of troponin-T is regulated by ASF/SF2, but not RBM20 (refs. 2,4,12), and RBM20 apparently regulates a larger number of cardiac proteins than SC35 or ASF/SF2 (refs. 2,11,12). Numerous human *RBM20* point mutations have been found that cluster in exon 9 of the gene, which encodes the RS domain[2,3,6] that can interact with both RNA and protein. Thus, it is conceivable that alterations in the propensity of the RS domain to bind RNA or other splicing factors adversely affect the spliceosome and may underlie the anomalous isoform patterns in *RBM20*-mutant hearts.

Interestingly, ASF/SF2 and RBM20 were previously considered as potential candidate factors involved in regulating the reversion from the adult to the fetal cardiac gene expression program that has been observed in heart failure[13]. At least for RBM20, this suggestion does not hold up. Using titin as a paradigm, Guo *et al.*[2] show that previously undescribed patterns of titin isoforms are expressed in both rat *Rbm20*- and human *RBM20*-mutant hearts. For instance, *Rbm20*-mutant rats express the giant N2BA-G titin variant, which is not detectable in other normal mammalian (including human) fetal and adult hearts, not even when they are in end-stage failure. Furthermore, the lack of N2B titin in human adult hearts with an *RBM20* mutation has not been observed in other adult mammalian hearts, regardless of whether they are normal or failing. As *RBM20* mutations do not grossly interfere with heart development and do not cause fetal protein isoforms to be re-expressed in adults, it remains intriguing what determines the transition between fetal and adult cardiac gene programs. Although this issue and others remain to be resolved in further studies, the work of Guo *et al.*[2] confirms and extends the emerging concept that human cardiomyopathies can be triggered by dysfunctional RNA processing.

## COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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# Horizontal gene transfer boosts MRSA spreading

André Kriegeskorte & Georg Peters

**Mechanisms triggering methicillin-resistant *Staphylococcus aureus* (MRSA) epidemics are poorly understood. A recent study provides new evidence that horizontal gene transfer may be the culprit for the emergence of new resistant and virulent MRSA clones.**

*S. aureus* is one of the major human pathogens worldwide, causing a wide range of diseases ranging from mild skin infections to life-threatening invasive infections such as pneumonia, endocarditis and sepsis[1]. Although *S. aureus* belongs to the normal human flora (nose and skin) and colonizes approximately 30% of the healthy population without causing any symptoms, it has been shown that nasal carriage is a crucial risk factor for serious invasive *S. aureus* infections[2–4]. The epidemiological situation of *S. aureus* today has been considerably shaped by selective pressure caused by the introduction of β-lactam antibiotics. The introduction of penicillin in the 1940s led to the selection of resistant strains with a plasmid-borne β-lactamase, an enzyme that can hydrolyze the peptide bond of the β-lactam ring of penicillins, leading to inactivation of the drug. These strains became pandemic in the 1950s. Shortly after the introduction of methicillin—β-lactamase–resistant penicillin—in 1959, MRSA strains emerged in the UK[5] and began to spread worldwide.

This β-lactam antibiotic resistance is based on the acquisition of a mobile genetic element, called staphylococcal cassette chromosome *mec* (SCC*mec*), that contains *mecA*, which encodes an alternative penicillin-binding protein (PBP2a, a transpetidase) that compensates for the function of the native methicillin-sensitive PBP2 (ref. 6). The high diversity of this element and the occurrence of different SCC*mec* types in the same MRSA clonal lineage led to the suggestion that these elements were acquired independently at several times through horizontal gene transfer (movement of genetic information between bacteria independently of the replication process). The evolutionary origin and the detailed mechanism of transfer of this element is still unclear, but there is good evidence that SCC*mec* elements were transferred from coagulase-negative staphylococci to *S. aureus*[7].

Today, the increasing dissemination of MRSA in hospitals, in the community and in livestock is a major threat to healthcare systems worldwide, as MRSA infections increase morbidity, mortality and socioeconomic costs[7]. Additionally, the rapid acquisition of other types of antibiotic resistances (beside β-lactam resistance) increasingly restricts therapeutic options. Currently, mainly glycopeptides and lipopeptides, such as vancomycin and daptomycin, respectively, are the last line of defense against MRSA. Studies using modern molecular approaches such as *S. aureus* protein A gene (*spa*) typing[8], multilocus sequence typing[9] and even whole-genome sequencing[10] have given insight into the MRSA epidemiology. As a result, today five distinct MRSA clonal complexes have been shown to be predominant around the world[11]. Nevertheless, the local MRSA population structure can differ substantially and is only characteristic for specific geographical regions, cities, hospitals or even single intensive-care units. These structures of MRSA strains are anything but rigid—they are permanently evolving, influenced by various factors only insufficiently understood. In this context, horizontal gene transfer as a main driving force of bacterial evolution has been extensively stressed, but studies supporting the concept are rare.

In this issue of *Nature Medicine*, Li *et al.*[12] show substantial new evidence that the acquisition of a new virulence determinant occurs through horizontal gene transfer and that this could be the trigger for the occurrence of new successful clones. The study identified a staphylococcal surface protein—SasX—to be epidemiologically connected to a successful quickly spreading clone of the MRSA sequence type 239 (ST239) in three hospitals in eastern China.

This commonly hospital-associated sequence type is ubiquitously disseminated and predominant in Asia, Eastern Europe and South America[9]. SasX was first identified in a strain (TW20) from an MRSA outbreak in an intensive-care unit in the UK[13], and whole-genome sequencing revealed that it is located on a mobile genetic element (ΦSPβ-like prophage) 127.2 kb in size (**Fig. 1**). SasX shows high homology to SesI (also encoded by ΦSPβ prophage) of *S. epidermidis* RP62A, which is supposed to increase its invasive capacity[10]. Li *et al.*[12] showed that *sasX* is rare within a

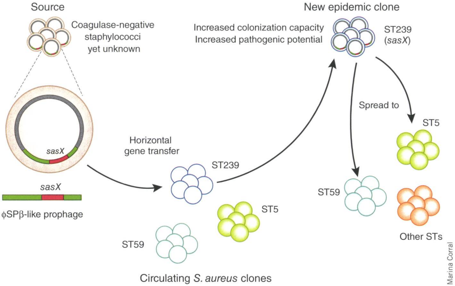

**Figure 1** Schematic view of the development of new epidemiologically successful clones triggered by horizontal gene transfer. Li *et al.*[12] show that *sasX*, encoded on a mobile genetic element, is a potent virulence determinant enhancing the colonization capacity and pathogenic potential of *S. aureus* and has the potential to spread to other sequence types (STs).

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representative global collection of ST239 isolates from divergent geographical backgrounds and absent in all other major global MRSA clones. Investigating the epidemiological situation in three large teaching hospitals in eastern China over an 8-year period, the authors showed a significant increase of *sasX*-positive isolates within the MRSA population[12]. Interestingly, the *sasX*-containing element (initially restricted to ST239) seemed to spread to other sequence types (**Fig. 1**), which could indicate an increased mobilization capacity of this element within the hospital setting in eastern China.

Li *et al.*[12] went on to further characterize the biological role of SasX by knocking it out in two different *S. aureus* genetic backgrounds in several *in vitro* and *in vivo* settings. SasX, an LPXTG surface-anchoring motif–containing protein, was expressed on the surface of bacterial cells, and it was shown to have an impact on the colonization capacity and pathogenicity of *S. aureus*. The authors found that SasX promotes adhesion to human nasal epithelial cells and increases nasal colonization in two mouse colonization models[12]. Furthermore, SasX facilitated interbacterial aggregation and biofilm formation, leading to reduced phagocytosis and increased survival in human blood and resistance to human neutrophils. Two mouse infection models, an abscess model and a lung infection model, revealed a strong impact of SasX on *S. aureus* pathogenicity. The increased adhesion and aggregation properties mediated by SasX may enhance the immune evasion capacity of *S. aureus* ST239, facilitating the transmission and spread of this clone in the clinical setting and making this clone more successful than others.

On the basis of the epidemiological data and on how SasX functions in *S. aureus*, Li *et al.*[12] proposed that horizontally acquired virulence determinants could serve as an important trigger for the occurrence of new epidemiologically successful or epidemic strains. Finally, they noted that *sasX* has the potential to spread throughout Asian and international *S. aureus* MRSA clones[12], which entails the risk of an increased colonization capacity and pathogenic potential of these strains.

However, one may assume that a virulence determinant such as SasX is only one factor out of several factors that can influence the success of MRSA clones; the complex interplay of internal factors, including genetic background and horizontal acquired determinants, and external factors, such as local hygiene measures, antibiotic usage, human and livestock population structure and potential transmission pathways, that influence epidemiological success should be considered. Detailed knowledge of the whole genomic background of epidemic strains is a prerequisite for the comprehensive understanding of the molecular mechanisms leading to epidemiological success. Not only single genes—but also the interplay of several factors—have to be considered. Indeed, there is good evidence that SasX serves as the main driving force of the Chinese ST239 MRSA epidemic; however, questions concerning its role in other MRSA sequence types such as ST5 that are also highly prevalent in this region and in methicillin-sensitive *S. aureus* (MSSA) clones remain and have to be assessed in future studies. Recently, a study showed the occurrence of an ST239 subtype harboring an arginine catabolic mobile element (ACME)—potentially acquired by horizontal gene transfer—that may have contributed to the success of the clone in a clinical setting in Sydney[14].

The findings of Li *et al.*[12], together with previous evidence, underline that horizontal gene transfer is a major mechanism driving the epidemiological success of MRSA clones. A worldwide population-based surveillance of MRSA population structure is needed to get awareness of new epidemic lineages at an early stage of dissemination and to effectively control new MRSA epidemics. It may even become increasingly important to monitoring not only MRSA but also MSSA and other coagulase-negative staphylococci, which could serve as a reservoir for new virulence factors with the inherent potential to boost *S. aureus* epidemicity.

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The authors declare no competing financial interests.

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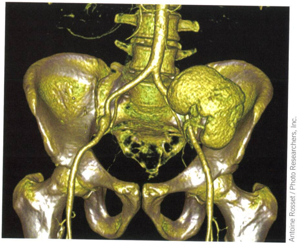
# COMMUNITY CORNER

# A step closer to effective transplant tolerance?

**Inducing recipient tolerance to organ or tissue transplants mismatched for major histocompatability complex (MHC) proteins still represents a formidable challenge. Previous studies have indicated that mixed hematopoietic chimerism may be one route to achieving stable transplant tolerance. Leventhal *et al.*[1] recently carried out human leukocyte antigen (HLA)-mismatched kidney transplants in eight people using a strategy that incorporated nonmyeloablative conditioning with a bioengineered cellular therapeutic enriched for hematopoietic stem cells, which was followed by post-transplant immunosuppression. This protocol achieved a high degree of durable donor chimerism, and five of the eight patients could be weaned off all immunosuppression 1 year after the transplant. We asked four experts to comment on the results of this study.**



Antoine Rosset / Photo Researchers, Inc.

## Laurence A Turka & Jeffrey A Bluestone

In 1960, Medawar and Burnet received the Nobel Prize in Physiology or Medicine for their discovery of acquired immune tolerance. Many felt that immune tolerance would soon be achieved in the clinic, solving the problem of organ graft rejection, but this remains an elusive goal. Although it is relatively easy to induce transplantation tolerance in rodents, few approaches have shown success in people, partly due to the robust alloimmune response. Thus, effective tolerance induction requires elimination of alloreactive cells and establishment of a noninflammatory environment, which promotes active immune regulation in the context of graft antigens. So far, only the induction of mixed chimerism via bone marrow transplantation (BMT) or stem cell transplantation, pioneered by Ildstad and Sachs almost 30 years ago[2], has worked in humans.

Recent successes using BMT or stem cell transplantation to induce tolerance in HLA-matched live donor renal allografts[3,4] have led to protocols for HLA-mismatched transplantation, a much more stringent setting, but one with greater clinical applicability. However, translating this approach will require overcoming several difficulties. The regimens require substantial myeloablation, which is more intensive than the one normally used for routine immunosuppression in solid-organ transplant recipients, raising concerns of safety and adaptability to nontertiary care facilities. BMT across HLA barriers is associated with the risk of bone marrow rejection and also with the risk of graft-versus-host disease (GVHD, a complication that is generally deemed unacceptable by physicians). Thus, transplantation protocols must strike a delicate balance.

Sachs and his colleagues previously reported long-term, drug-free renal allograft survival without severe GVHD or immune deficiency in two clinical trials[5]. However, most patients experienced early transient graft dysfunction due to an 'engraftment syndrome'; two patients lost their grafts and a third has severe dysfunction. Several groups are engaged in modified approaches that may avoid these complications, including the use of conditioning regimens that include alloantigen-bearing cells; for example, a recent trial used a proprietary donor cell product to expose the host to potentially tolerogenic alloantigen[1].

Compounding the complications for translational studies is our incomplete understanding of how tolerance is induced via BMT. Although studies in mice

## Herman Waldmann

A major challenge in organ transplantation is minimizing drug immunosuppression by harnessing mechanisms of immunological tolerance. Full donor chimerism using lethal irradiation[8] and nonmyeloablative[9] radiation has been shown in rodents. This chimerism is stable and accompanied by tolerance to transplanted tissues from the allogeneic donor. Concerns about the immune competence of T cells that develop in a completely MHC-mismatched thymus led Ilstadt and Sachs to propose mixed (host plus donor) chimerism as a route to transplantation tolerance[2]. Full-donor chimerism can generate tolerance with a minor need for active immune regulation in rodents, whereas although low levels of donor chimerism allow 'operational' tolerance, this is achieved through a more substantial role for regulatory T cells[10].

Leventhal *et al.*[1] have now shown that a substantial proportion of transplant recipients given myelo- and immunosuppressive irradiation and drugs, together with a cell product that facilitates donor stem cell engraftment,

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have suggested long-term chimerism is needed for tolerance, the data in humans are far less clear. At what level and for how long will chimerism be required to develop robust tolerance and allow drug withdrawal? If immunoregulation is the key to the 'maintenance' phase of tolerance, then stable mixed chimerism may be irrelevant, and trying to achieve it could place undue safety burdens on the patient. Therefore, assays to assess a patient's immune status are needed, as a drug treatment for autoimmunity or transplantation is not about maximal tolerated dose but finding the right balance needed for tolerance.

Paradoxically, perhaps, the biggest challenge to achieving tolerance may be the success of transplantation itself, at least in the short term. One-year renal allograft survival approaches 95%, and several protocols for immunosuppression minimization are quite effective. Many patients and their doctors feel satisfied with continued low-dose monotherapy and are loath to discontinue immunosuppression, as even a reversible graft rejection episode may lead to irreversible scarring and impairment of graft function. Thus, unless there are other compelling medical reasons for avoiding immunosuppression, tolerance protocols may not achieve acceptable equipoise for institutional review boards, physicians or patients. In this regard, liver transplantation is being investigated as a therapeutic model, given the protolerogenic nature of this organ and the ability of the liver tissue to repair following a rejection episode.

So what does that mean for future tolerance trials? Although studies in renal transplantation will (and should) continue, much effort is focusing on developing effective biomarkers for immune responsiveness and tolerance. Recent studies have proposed rejection and tolerance signatures based on serum or urine mRNA and/or miRNA profiling, and these are being tested prospectively[6,7]. The successes in inducing tolerance via BMT show that tolerance can be a reality and provide a road map to achieve the success first envisioned by Medawar, Burnet and the many physicians and scientists devoted to immune tolerance efforts.

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showed high levels of donor chimerism and could be weaned off immunosuppression. This achievement of substantial donor chimerism with a relatively low-stringency conditioning protocol is admirable.

Several questions remain. Were the 'facilitating cells' needed? How could such a protocol be applicable to cadaveric donors, in which case facilitating cells would have to be processed ahead of the transplant and fludarabine administered to the recipient some days before the operation? If the reconstituting T cells are donor derived, as observed in this study, is there a need for mixed chimerism at all? Is the mixed chimerism concept now irrelevant?

The lack of GVHD observed, which needs to be understood, provides the most encouraging aspect of this work. Looking to the future, though, I find it hard to imagine that a facilitating cell product would need to be part of any routine organ transplant procedure, for both logistical and commercial issues. The importance of this study is the demonstration that high donor chimerism can be achieved without GVHD through acceptable conditioning protocols.

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## Megan Sykes

Allograft tolerance obviates the requirement for long-term immunosuppressive therapy and prevents chronic rejection. Induction of mixed hematopoietic chimerism achieves robust allograft tolerance in rats and mice and is advantageous over full allogeneic chimerism because it can be achieved with less dangerous conditioning regimens and because it ensures immunocompetence. Immunocompetence is often compromised in full chimeras because they lack a supply of peripheral recipient antigen-presenting cells (APCs) to present antigens optimally to donor T cells that are positively selected on recipient thymic epithelial MHC molecules. In the absence of MHC sharing between donor and recipient, T cells do not efficiently recognize peptides presented by donor APCs, which are the only ones available in full chimeras.

An approach to tolerance induction through mixed chimerism that was translated successfully from rodents, through nonhuman primates and into humans in an intentional induction of renal allograft tolerance across HLA barriers for the first time was published 4 years ago[5]. A second approach, across even more extensive HLA barriers (including unrelated donors) has recently been reported[1]. The regimen, originally developed for the treatment of patients with cancer[11], involved fludarabine, low-dose total body irradiation and pre- and post-transplant cyclophosphamide to purge host-versus-graft and graft-versus-host reactive T cells. The 32% GVHD rate and 7% nonrelapse mortality[11] were considered acceptable in this challenging, HLA haplotype–mismatched, related-donor transplant protocol for treatment of hematological malignancies. However, a key safety criterion to be met before the use of hematopoietic cell transplantation solely to achieve organ allograft tolerance should be the absence of GVHD. GVHD was not reported in the five patients with durable engraftment of donor bone marrow in this allograft tolerance trial[1], and its absence was attributed to the co-infusion of facilitating cells, a cell type of uncertain lineage prepared by a proprietary method not disclosed in the publication and for which the authors declared financial interests. One patient suffered a near-lethal infectious complication and lost his kidney graft[1]. However, the restoration of immunocompetence in these fully allogeneic chimeras with extensively HLA-mismatched donors remains to be seen. No safety or efficacy data using this method in nonhuman primates have been reported. Thus, although the results are remarkable, the potential of this approach to safely induce allograft tolerance remains unclear.

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The author declares no competing financial interests.

# BETWEEN BEDSIDE AND BENCH

# Shedding pounds after going under the knife

**Losing weight can pose a challenge, but how to avoid putting those pounds back on can be a real struggle. A major health problem for obese people is that diseases linked to obesity, such as type 2 diabetes and cardiovascular disease, put their lives at risk, even in young individuals. Although bariatric surgery—a surgical method to reduce or modify the gastrointestinal tract—was originally envisioned for the most severe cases of obesity, evidence suggests that the benefit of this procedure may not be limited to the staggering weight loss it causes. Endogenous factors released from the gut, and modified after surgery, may explain why bariatric surgery can be beneficial for obesity-related diseases and why operated individuals successfully maintain the weight loss. In 'Bedside to Bench,' Rachel Larder and Stephen O'Rahilly peruse a human study with dieters who regained weight despite a successful diet. Appetite-regulating hormones in the gut may be responsible for this relapse in the long term. In 'Bench to Bedside,' Keval Chandarana and Rachel Batterham examine how two different methods of bariatric surgery highlight the relevance of gut-derived hormones not only in inducing sustained weight loss but also in improving glucose homeostasis. These insights may open new avenues to bypass the surgery and obtain the same results with targeted drugs.**

### BEDSIDE TO BENCH

## Guts over glory—why diets fail

Rachel Larder & Stephen O'Rahilly

The epidemic of obesity is now a major public health concern in many parts of the world, given its impact on diabetes, heart disease, stroke and cancer[1]. Whereas genetics play a part in determining an individual's propensity to gain weight[2], the increased prevalence of obesity is likely to be attributable to a global increase in the consumption of energy-dense foods alongside a substantial decrease in physical activity levels. Although surgical methods such as gastric banding, sleeve gastrectomy and Roux-en-Y gastric bypass offer an effective treatment for weight loss (average weight loss of 62.1% 2 years after surgery[3]), they are generally recommended only for the morbidly obese, owing to the risk of postoperative mortality. For most obese individuals, the simplest way to reduce adiposity is to enact lifestyle changes that will result in weight loss, including increasing exercise levels and decreasing caloric intake. Although most dieters will initially see promising results within the first few months of embarking on a weight-loss program, the majority of them will have regained almost all of their original weight loss within 5 years[4]. A recent study in *The New England Journal of Medicine*[5] helps shed light on why this high 'relapse' rate occurs and suggests that appetite-regulating hormones produced by the gut may sabotage the good intentions of long-term dieters.

Regulation of body weight is a highly complex process centrally regulated within the brain[6]. Multiple hormonal signals from the gut, pancreas and fat are secreted in response to caloric intake and signal to the hypothalamus via neuronal or endocrine pathways to either promote or inhibit appetite[6]. Satiation signals such as glucagon-like peptide-1 (GLP-1), cholescystokinin (CCK) and peptide YY (PYY) are secreted by the intestine in response to food, with the aim of inhibiting appetite. In contrast, ghrelin secretion from the stomach is decreased after a meal, when appetite no longer needs to be stimulated. In addition, the adipocyte hormone leptin provides a longer-term signal to the brain regarding the status of energy stores within fat cells, whereas gastric inhibitory polypeptide (GIP) release from the intestine controls post-prandial insulin secretion and promotes energy storage[7].

When an individual's calorie intake is substantially reduced, the immediate response of the body is to defend against potential weight loss to ensure that reproductive integrity and survival will not be compromised—by dramatically increasing ghrelin and GIP secretion while decreasing PYY, CCK and GLP-1 levels in the circulation. The resulting increased appetite should initiate the immediate intake of calories so that energy homeostasis, and therefore fat deposits, can be maintained (**Fig. 1**).

Given the poor long-term success of many diets[4], Sumithran *et al.*[5] hypothesized that these appetite-driving hormonal changes are more than just a transient compensatory response and might persist beyond the initial 'defensive' response period. This would result in a continued increase in appetite and may explain the high relapse rate seen in many long-term dieters. The study reports the findings in 36 compliant obese participants in a weight-loss study involving an initial 8-week period of meal replacement with a very-low-calorie meal-replacement diet. During weeks 9 and 10, these participants, all of whom had lost 10% or more of their initial body weight, were gradually reintroduced to ordinary foods, and weight was stabilized. At the end of week 10, participants received individual counseling and written advice from a dietician on a dietary intake that would be consistent with their calculated energy expenditure, with the aim of weight maintenance. Satiety and hunger hormones were measured at three time points: before weight loss, after 10 weeks of weight loss and 1 year after the initial 10% reduction in weight.

Although the study was able to recapitulate

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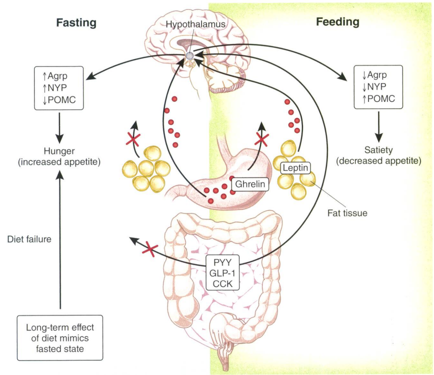

**Figure 1** Effect of feeding and fasting on hunger hormones. During and after regular feeding, there is a reduction in the production of ghrelin by the stomach. In contrast, production of PYY, GLP-1 and CCK from the gut is increased, and serum leptin levels also rise. These changes are detected by the brain, resulting in modulation of gene expression of orexigenic (Agouti-related protein (AGRP) and neuropeptide Y (NPY)) and anorexigenic (pro-opiomelanocortin (POMC)) proteins, which result in decreased appetite and a feeling of satiety such that caloric intake is stopped. During fasting, decreased food intake suppresses the release of PYY, GLP-1 and CCK from the gut while stimulating the secretion of ghrelin by the stomach. Fasting also decreases serum leptin levels. These changes are detected by the brain, resulting in upregulation of orexigenic and downregulation of anorexigenic gene expression within the hypothalamus, leading to hunger. Dieting results in a gut hormone profile that mimics the fasted state. These changes can persist for 12 months after initial weight loss and may explain why the majority of obese dieters fail to maintain a reduced body weight.

previously published data showing the expected short-term changes in various appetite-regulating hormones after 10 weeks of weight loss (**Fig. 1**), the data obtained 1 year after weight loss also showed that circulating mediators of appetite never returned to pre-intervention levels. At the last time point measured, the subjects continued to show substantially decreased levels of leptin, PYY and CCK and increased levels of ghrelin and GIP, coupled with a regain of about 30% of the weight they had lost a year before. The decreased leptin levels observed in the subjects were not unexpected, as some fat loss was maintained. Yet, the persistent changes in circulating gut hormone levels and increased hunger 12 months after initial weight loss were unexpected. Taken together with recent data showing that the reduction in energy expenditure seen immediately after weight loss can also endure for more than 12 months[8], these data present a strong physiological basis for the high rate of recidivism to obesity after nonsurgical approaches to weight loss.

Currently, bariatric surgery incorporating gastrointestinal bypass is the only obesity treatment that routinely achieves substantial and permanent weight loss[9]. Interestingly, recent research focusing on this success has shown that, unlike dieting, bariatric surgery is associated with marked appetite-suppressing changes in ghrelin, PYY and GLP-1 abundance, which presumably dominate over the weight-gain–promoting effects of lowered plasma leptin concentrations[10]. Although the mechanisms of these changes are not fully understood, it is thought that by bypassing a substantial portion of the small bowel, and thus increasing delivery of unabsorbed nutrients to the PYY- and GLP-1–producing cells of the distal bowel, an amplified gut-hormone response to food is achieved, resulting in increased satiety after surgery.

Given that rodent studies suggest that increased PYY levels are crucial for the maintenance of weight loss after gastric bypass surgery[11,12], studies of gut hormone profiles in the small percentage of subjects that fail to maintain weight after gastric bypass would be helpful in determining whether increased ghrelin and decreased PYY and GLP-1 levels contributed to their relapse and whether they are really essential for weight loss. More research is also warranted into how changes in these gut hormones after bypass surgery contribute to the rapid resolution of type 2 diabetes, often before substantial weight loss is observed. GLP-1 receptor antagonists blocked improvements in oral glucose tolerance tests in rats that underwent bypass surgery, suggesting that post-operative increases in GLP-1 are key for improving glucose homeostasis after bariatric surgery[13].

The report by Sumithran *et al.*[5] highlights the importance of afferent signals from the gastrointestinal tract to the brain in the maintenance of energy homeostasis and suggests that a potential therapy for obesity might be developed if one can replicate the hormonal milieu seen after gastric bypass surgery. With both leptin and ghrelin failing to show great promise as antiobesity drug targets, current focus has shifted to the development of drugs based on the function of specific gut hormones such as GLP-1 and PYY[14]. With nausea proving to be a common adverse effect during clinical trials of these drugs, the successful achievement of an anorectic effect with these drugs will probably depend on developing sustained-release formulations of a low-dose cocktail of multiple gut hormones that can be dispensed together to match the benefits of gastric bypass surgery without having to go under the knife.

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The authors declare no competing financial interests.

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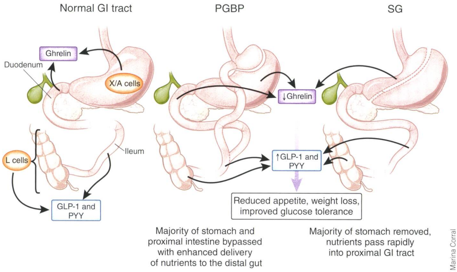
■ BENCH TO BEDSIDE

# Metabolic insights from cutting the gut

**Keval Chandarana & Rachel L Batterham**


Normal GI tract
PGBP
SG
Ghrelin
Duodenum
X/A cells
↓Ghrelin
L cells
Ileum
GLP-1 and PYY
↑GLP-1 and PYY
Reduced appetite, weight loss, improved glucose tolerance
Majority of stomach and proximal intestine bypassed with enhanced delivery of nutrients to the distal gut
Majority of stomach removed, nutrients pass rapidly into proximal GI tract

Marina Corral

**Figure 1** Hormonal insights from bariatric surgery. Several hormones are released from the gastrointestinal (GI) tract into the circulation (left); the 'hunger hormone' ghrelin is secreted from X/A cells, whereas PYY and GLP-1 are secreted from L cells, with the greatest concentration of L cells in the ileum and colon. In proximal gastric bypass (PGBP) (middle), a small gastric pouch is created and anastomosed with the mid-jejunum. Anastomosis of biliopancreatic limb with the jejunum allows drainage of biliary and pancreatic secretions. Nutrients flow through the gastric pouch and directly into the mid-jejunum, therefore bypassing the majority of the stomach, the duodenum and the proximal jejunum. In sleeve gastrectomy (SG) (right), the stomach is transected along the greater curve and the entire gastric fundus and body are removed without manipulation of small bowel. Nutrients enter the gastric sleeve and subsequently the small intestine with normal secretion of biliary and pancreatic products. Both PGBP and sleeve gastrectomy seem to result in reduction of circulating ghrelin and increased levels of GLP-1 and PYY, despite distinct anatomical differences. This combination of changes in the hormonal milieu might account for the improved glucose control and reduced appetite with subsequent weight loss observed after both procedures.

Bariatric surgery is currently the most effective treatment option for obesity, resulting in substantial and durable weight loss with reduction in mortality and obesity-related co-morbidities[1]. Obesity is the primary risk factor for type 2 diabetes mellitus (T2DM), which is characterized by insufficient insulin secretion and insulin resistance of peripheral tissues[2]. Proximal gastric bypass surgery (PGBP) is considered the 'gold standard' bariatric procedure, reducing excess body weight by up to 80%. Additionally, the majority of individuals with T2DM that undergo PGBP show immediate improved glycemic control after surgery, before significant weight loss. Although many therapeutic regimens aim to modulate glucose concentrations to prevent end-organ damage, bariatric surgery seems to be the only treatment modality with the potential to induce complete resolution of T2DM.

The mechanisms underlying this process, however, remain elusive. Recently, a technically less complex operation, sleeve gastrectomy, has been shown to result in weight loss and enhanced glycemic control comparable to PGBP[3]. Understanding how these surgical procedures induce sustained weight loss and resolution of T2DM holds the key to development of more directed, less invasive therapies. In a recent study, Chambers *et al.*[4] investigated bariatric procedures in rodent models of human obesity, highlighting the comparable effectiveness of sleeve gastrectomy with PGBP and the importance of gut-derived hormones in mediating improved glucose homeostasis after surgery.

PGBP restricts stomach volume by creation of a gastric pouch of approximately 20 ml along the lesser curvature that is anastomosed with mid-jejunum; hence, nutrient flow is rerouted from the upper stomach rapidly into the mid-jejunum and, subsequently, the unaltered distal gastrointestinal tract. Accordingly, most of the stomach, the entire duodenum and the proximal jejunum are bypassed but remain *in situ*. PGBP was designed to restrict gastric volume, limiting food intake and reducing nutrient absorption to induce weight loss. Sleeve gastrectomy was intended to be a solely restrictive procedure; it involves partitioning and removal of 80–90% of the stomach, parallel with the lesser curvature without altering the anatomy of the small intestine (**Fig. 1**). Sleeve gastrectomy therefore leads to rapid emptying of gastric contents into the small bowel[5]. Data from patient populations concerning long-term outcomes of sleeve gastrectomy are limited, but findings to date illustrate comparable efficacy with PGBP, with excess weight loss of up to 75% and resolution in T2DM in up to 84% of cases[6,7]. In a recent position paper, the International Diabetes Federation supported the selective use of various bariatric procedures for obese individuals with medically resistant T2DM[2].

The means by which these procedures achieve such impressive outcomes seems to involve alterations in several gut-derived hormones including ghrelin, peptide YY (PYY) and glucagon-like peptide 1 (GLP-1)[6]. Ghrelin is the only orexigenic hormone identified to date; it is mainly produced by the endocrine X/A secretory cells within the stomach fundus and duodenum. Diet-mediated weight loss results in increased circulating ghrelin concentrations[8]; in contrast, several studies report low circulating ghrelin concentrations after PGBP[6]. Although the gastric fundus remains *in situ*, nutrient contact with ghrelin-secreting cells is interrupted. Sleeve gastrectomy, which involves complete removal of the majority of the gastric fundus and hence the major population of ghrelin-secreting cells, also leads to substantial and sustained reductions in ghrelin concentrations[9]. Together, these observations indicate that disruption of nutrient stimulation of endocrine cells within the stomach contributes to the beneficial effects of these modes of bariatric surgery.

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PYY is an anorectic hormone that is secreted from enteroendocrine L cells, which are located throughout the gastrointestinal tract but mostly concentrated in the ileum and colon. After a meal, plasma PYY concentrations rise proportionally to caloric intake—although this response is blunted in obese subjects[10].The L cells also produce the incretin hormone GLP-1, which stimulates insulin secretion. Additionally, GLP-1 is thought to promote insulin sensitivity and may contribute to appetite suppression after eating. Several pharmacological agents targeted at the GLP-1 axis are being used to treat T2DM, including GLP-1 receptor agonists and dipeptidyl peptidase IV (DPP4) inhibitors, which extend the half life of biologically active endogenous GLP-1 (ref. 11). Several studies in humans show that PGBP leads to concomitant increases in both PYY and GLP-1 after a meal, with rises in these hormones occurring early after surgery[12] and independently of weight loss[13]. Early studies investigating gut hormone changes after sleeve gastrectomy suggest similar increases in post-operative nutrient-stimulated plasma PYY and GLP-1 concentrations[12].

It is thought that increased circulating PYY levels after surgery contribute to appetite reduction and hence drive weight loss, whereas increased circulating GLP-1 levels account for the marked changes in glucose control. Interestingly, the use of pharmaceutical GLP-1 agonists and DPP4 inhibitors alone does not result in the same glycemic benefits or T2DM resolution seen after bariatric surgery. Moreover, the changes in circulating levels of ghrelin, PYY and GLP-1 occur in synchrony post-operatively, indicating that the distinct roles of each hormone require delicate investigation using appropriate models. To account for the hormonal alterations induced by bariatric surgery, the 'foregut theory' was proposed, suggesting that bypassing the duodenum and proximal jejunum removes an anti-incretin factor, leading to improved glucose control[14]. However, the observed similarity in weight loss and glycemic control after sleeve gastrectomy, which does not entail bypass of the duodenum and proximal jejunum, brings this theory into question. The alternative 'hindgut theory' suggests that increased flux of unabsorbed, incompletely digested nutrients to the distal gastrointestinal tract causes greater L cell stimulation and therefore increased circulating GLP-1 and PYY concentrations.

To further understand the dynamic alterations in gut hormones engendered by surgery, animal models of PGBP and sleeve gastrectomy have been developed. In their recent study, Chambers *et al.*[4] performed these bariatric procedures on diet-induced obese rats to investigate putative mechanisms underlying altered glucose handling in the early post-operative period. The two procedures resulted in similar improvement in insulin sensitivity and glucose tolerance as well as similar concentrations of meal-stimulated GLP-1 secretion in the operated obese rats compared to sham-operated control rats. Moreover, the studies showed that blocking GLP-1 activity with an antagonist of its receptor (exendin9-39) blunted surgically induced improvements in glucose tolerance and insulin secretion[4]. Whereas previous studies have strongly alluded to the role of GLP-1 in the glycemic improvements after bariatric surgery, this study strongly supports that enhanced GLP-1 concentrations directly lead to increased insulin secretion and glucose control after both procedures.

This study provides further evidence negating the importance of proximal duodenal and jejunal bypass. Furthermore, it suggests that sleeve gastrectomy induces changes in the hormonal milieu akin to PGBP and may enhance nutrient delivery to the distal gastrointestinal tract without intestinal bypass. Modified gastric bypass procedures in diet-induced obese and *Pyy*-deficient obese mice have confirmed a key role of PYY in weight loss after surgery, as mice lacking PYY did not show the substantial weight loss observed in diet-induced obese mice early after surgery[15]. Current investigations in gut-derived cell lines from mouse models, employing electrophysiology studies, are providing further insights into L cell secretion mechanisms[16].

Bariatric surgery represents a safe treatment modality for sustained weight loss but involves procedural variability. Data from a large cohort of subjects suggest that 30-d mortality after bariatric procedures is low (less than 0.3%)[7,17]; however, a number of negative outcomes have been reported, including anastomotic leaks and macro- or micronutrient deficiencies[1]. Developing less invasive methods for weight loss, with efficient and durable outcomes, while minimizing complication rates, is a key focus of current investigations. Further studies are crucial to elucidate precise mechanisms underlying the metabolic benefits of bariatric procedures. Such studies will ensure the development of nonsurgical means to induce favorable hormonal profiles and sustainable weight loss to reduce obesity-related morbidity and mortality.

### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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**METABOLISM**

## Calcium-mediated control

Glucose production by the liver is key to the maintenance of glucose homeostasis and is regulated by the actions of hormones such as insulin and glucagon. Two new studies uncover details of how glucagon regulates glucogenic transcription factors, highlighting a role for calcium signaling in hepatic glucose homeostasis.

Previous studies had linked intracellular calcium to regulation of gluconeogenesis, so Lale Ozcan *et al.* (*Cell Metab.* doi:10.1016/j.cmet.2012.03.002) focused on the calcium-sensing enzyme calcium calmodulin-dependent kinase II (CaMKII). They showed that this enzyme is activated by cyclic AMP (cAMP) and glucagon in a calcium-dependent and inositol 1,4,5-triphopshate receptor ($IP_3R$)-dependent manner in hepatocytes. $IP_3R$ channels in the endoplasmic reticulum release calcium in response to ligand binding and are important in calcium homeostasis. CaMKII was also activated in the livers of mice in response to glucagon and fasting. A mouse knockout of CaMKIIγ had defective hepatic glucose production and failed to show the phosphorylation and nuclear translocation of the glucogenic transcription factor FoxO1 observed when wild-type mice were subjected to fasting. By contrast, a constitutively active CaMKII mutant had the opposite effects.

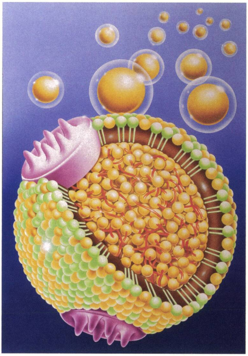
Ozcan *et al.* then examined the CaMKII pathway in two mouse models of obesity and found that the amounts of phospho-CaMKIIγ were higher in the livers of the obese mice than in those of wild-type mice. Genetic knockdown of CaMKII improved the metabolic deficits of the obese mice, lowering fasting glucose concentrations and the expression of FoxO1 target genes.

A second study by Yiguo Wang *et al.* (*Nature* doi:10.1038/nature10988) identifies how glucagon can affect the nuclear localization of another important glucogenic transcription factor, CRTC2, through a different calcium-sensing enzyme, calcineurin. This mechanism was also $IP_3R$ dependent, and the authors showed that hepatic calcineurin activity was increased in two mouse models of diabetes and that knockdown of calcineurin or $IP_3R$ could improve circulating glucose concentrations in these mice.

Together, these studies provide new mechanistic insights into the regulation of hepatic glucose production, and their findings may also be relevant in the settings of obesity and diabetes, although first it will be important to see whether these regulatory mechanisms also operate in humans. —*MS*

**CARDIOVASCULAR DISEASE**

## Lipoprotein traffic control



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**Insulin signaling in the liver affects lipid synthesis and uptake in many ways. Two new papers by Ai *et al.* in the *Journal of Clinical Investigation* show how an insulin-regulated signaling protein complex, mTORC1, acts to promote both lipoprotein secretion and uptake (doi:10.1172/JCI61248; 122, 1262–1270).**

**In the first paper, the authors showed in genetic or dietary mouse models of obesity that mTORC1 promotes secretion of apoB, the major apolipoprotein of the atherogenic lipoproteins very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL). Activation of hepatic mTORC1 triggers an endoplasmic reticulum stress response, and the researchers found that an effector of this pathway, the transcription factor Atf3, directly represses expression of the protein sortilin-1. This protein is involved in the intracellular trafficking of secreted proteins from the Golgi to lysosomes. It has previously been linked by genome-wide association studies in humans to LDL cholesterol levels and the risk of myocardial infarction and has also been reported to negatively regulate hepatic VLDL secretion in mice.**

**In the second paper, the authors showed that the effect of mTORC1 on lipoprotein uptake goes through a separate pathway involving the protein PCSK9, whose expression mTORC1 controls by signaling through the transcription factor HNF-1α. PCSK9, which like sortilin-1 is involved in intracellular protein trafficking, is thought to divert LDL receptor recycling into an endosomal-lysosomal pathway that leads to LDL receptor degradation. PCSK9 is known to affect LDL receptor function in humans: individuals with *PCSK9* mutations have high LDL receptor levels and low LDL cholesterol levels. —*MB***

**BRAIN**

## Bouncing back after brain injury

Changes in neural circuitry after brain injury can help the nervous system to heal. In a recent study, researchers have demonstrated that molecules known for their functions in the immune system can constrain brain plasticity after stroke (*Neuron* **73**, 1100–1107).

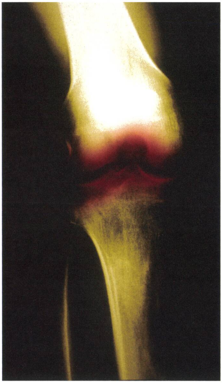
Major histocompatibility class I (MHCI) molecules present antigens on the surface of cells to stimulate the immune system, and paired immunoglobulin-like receptor B (PirB) is a receptor for MHCI molecules. MHCI and PirB are both expressed on neurons and are known to have a role in neural plasticity during development, so Jaimie D. Adelson *et al.* asked whether they could also be targeted to induce functional recovery after stroke.

The researchers found that stroke increased the expression of two MHCI subtypes as well as PirB in the mouse brain; mice lacking these proteins had better functional outcomes and less brain damage after stroke. This improvement seemed to be linked to an increase in axon projections from the uninjured cortex of these mice to the affected side of the body, although exactly how the MHCI molecules and PirB receptor affect this process remains to be determined. —*EC*

**BONE**

## Repairing joints

Osteoarthritis afflicts most adults over 55 and is caused by the progressive degeneration



Scott Camazine / Photo Researchers, Inc.

of cartilage at bone joints, for which there is no cure. Kristen Johnson *et al.* have now identified a small molecule with effects on mesenchymal stem cells (MSCs) that reduced the severity of disease in two rat models of osteoarthritis. The findings suggest new avenues for the development of drugs or stem cell therapies to combat this disease (*Science* doi:10.1126/science.1215157).

Chondrocytes are cartilage-resident cells that can build and repair cartilage and are generated from MSCs. Thus, directing the differentiation of MSCs into chondrocytes to stimulate joint repair could be a potential therapeutic strategy for osteoarthritis. Using this rationale, Johnson *et al.* screened 22,000 compounds and found that only one, kartogenin, could promote the differentiation of primary human MSCs into chondrocytes. When injected into the joints of rats following surgical or collagenase-induced damage, kartogenin restored cartilage and joint function. By coupling a derivative of kartogenin to biotin and a photoactivatable crosslinker and analyzing the MSC proteins bound to this kartogenin construct after ultraviolet irradiation, the researchers identified filamin A as the target of kartogenin. Filamin A crosslinks actin filaments, but this function was unaffected by kartogenin. Instead, the authors found that the kartogenin-binding region of filamin A also binds core-binding factor-β (CBF-β), the regulatory subunit of the CBF-β–RUNX transcription factor complex. They showed that kartogenin disrupts the binding of filamin A to CBF-β, resulting in the translocation of CBF-β to the nucleus, where it can then bind RUNX and regulate the transcription of chondrogenic genes. Silencing of CBF-β or RUNX1 inhibited the ability of kartogenin to differentiate MSCs into chondrocytes.

The RUNX proteins have previously been implicated in chondrocyte proliferation or differentiation, and the identification of kartogenin provides a new tool to regulate their activity by modifying the subcellular localization of their binding partner CBF-β. In addition, the study provides insight into the potential of MSCs to repair damaged joints that may help inform the development of future stem cell therapies for osteoarthritis in humans. —*AF*

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**■ IMMUNOLOGY**

## Targeting IL-17 in psoriasis

Two recent clinical trials provide evidence that interleukin-17 (IL-17) and its receptor are viable targets to treat psoriasis.

In one of these phase 2, randomized, double-blind trials, Kim Papp *et al.* (*N. Engl. J. Med.* **366**, 1181–1189) tested different doses of brodalumab, a monoclonal antibody against the IL-17 receptor A, or placebo in 198 patients with psoriasis. In a separate trial, Craig Leonardi *et al.* (*N. Engl. J. Med.* **366**, 1190–1199) treated 142 people with psoriasis with placebo or different doses of ixekizumab, a monoclonal antibody that directly targets IL-17.

After 12 weeks of treatment, both teams reported that the antibody-treated patients showed statistically significant improvements on the psoriasis area-and-severity index, a scale commonly used to quantify disease progression. Moreover, the safety profile of both antibodies was satisfactory.

Antibodies against other cytokines such as IL-12 and IL-23 are effective against psoriasis, acting at least in part by reducing the production of IL-17 and the generation of T helper type 17 immune responses. The clinical development of IL-17–targeting molecules may nevertheless be advantageous in terms of selectivity, as inhibiting the IL-12/IL-23 axis can also affect T helper type 1 responses, which are important in fighting infection. Phase 3 clinical trials will be necessary to establish the long-term safety and efficacy of brodalumab and ixekizumab. —*JCL*

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Written by Michael Basson, Eva Chmielnicki, Alison Farrell, Juan Carlos López and Meera Swami.

## New from NPG

**Regulation of circadian behaviour and metabolism by REV-ERB-α and REV-ERB-β**
Cho, H. *et al. Nature* doi:10.1038/nature11048 (29 March).

**Regulation of circadian behaviour and metabolism by synthetic REV-ERB agonists**
Solt, L.A. *et al. Nature* doi:10.1038/nature11030 (29 March).

These two studies disclose the contribution of the nuclear receptors REV-ERB-α and REV-ERB-β to the regulation of circadian rhythms and metabolic function, and provide evidence that this signaling pathway may be pharmacologically targeted.

**Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture**
Estrada, K. *et al. Nat. Genet.* doi:10.1038/ng.2249 (15 April).

The authors carried out a meta-analysis on lumbar spine and femoral neck bone mineral density, identifying 56 loci that showed association, of which 32 loci were new associations. The associated loci had functions that included RANK signaling, mesenchymal stem cell differentiation, endochondral ossification and Wnt signaling.

**NLRC4-driven production of IL-1β discriminates between pathogenic and commensal bacteria and promotes host intestinal defense**
Franchi, L. *et al. Nat. Immunol.* doi:10.1038/ni.2263 (8 April).

The authors provide new understanding of how the immune system discriminates between commensal and pathogenic bacteria. They show that resident intestinal phagocytes, when infected with pathogenic bacteria, produce the cytokine IL-1β through activation of the NLRC4 inflammasome. This pathway, which is important in the clearance of the pathogenic bacteria, is not activated when the phagocytic cells are infected by commensal bacteria.

**Tanycytes of the hypothalamic median eminence form a diet-responsive neurogenic niche**
Lee, D.A. *et al. Nat. Neurosci.* doi:10.1038/nn.3079 (25 March).

Adult neurogenesis in the hypothalamus has been reported, and these authors now show that new neurons are formed in a part of the hypothalamus called the median eminence. Notably, median eminence neurogenesis was increased in mice on a high-fat diet, and blocking the formation of these neurons reduced weight gain and increased energy consumption in mice, even in those on a high-fat diet.

# Almirall´s commitment to research in respiratory diseases

Almirall is a Barcelona-based international company that strives for innovation in the respiratory field. Treatment of airways diseases has been one of the major areas of development for Almirall and significant resources are dedicated to respiratory research. Almirall develops respiratory therapies with a focus on inhalation based therapies using our proprietary novel dry powder inhaler.

## Respiratory *pipeline*

| Therapy class | Preclin. | Phase I | Phase II | Phase III | Registration | Registration application |
|---|---|---|---|---|---|---|
| Antimuscarinic | 🟩 COPD | 🟩 | 🟩 | 🟩 | 🟩 | Submitted for registration in USA and EU |
| Antimuscarinic + LABA | 🟦 COPD | 🟦 | 🟦 | 🟦 | | Undisclosed |
| OD LABA + ICS | 🟧 Asthma / COPD | 🟧 | 🟧 | | | > 2014 |
| MABA | 🟪 COPD | | | | | > 2014 |

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nature medicine

# Innate and adaptive immune responses in asthma

Stephen T Holgate

**The recognition that asthma is primarily an inflammatory disorder of the airways associated with T helper type 2 ($T_H2$) cell-dependent promotion of IgE production and recruitment of mast cells and eosinophils has provided the rationale for disease control using inhaled corticosteroids and other anti-inflammatory drugs. As more has been discovered about the cytokine, chemokine and inflammatory pathways that are associated with $T_H2$-driven adaptive immunity, attempts have been made to selectively inhibit these in the hope of discovering new therapeutics as predicted from animal models of allergic inflammation. The limited success of this approach, together with the recognition that asthma is more than allergic inflammation, has drawn attention to the innate immune response in this disease. Recent advances in our understanding of the sentinel role played by innate immunity provides new targets for disease prevention and treatment. These include pathways of innate stimulation by environmental or endogenous pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) to influence the activation and trafficking of DCs, innate sources of cytokines, and the identification of new T cell subsets and lymphoid cells.**

## What is asthma?

Asthma is a disorder of the conducting airways leading to variable airflow obstruction in association with airway hyperresponsiveness (AHR). Airway inflammation is central to disease pathophysiology and relates to airway dysfunction partly through the release of potent inflammatory mediators and partly through remodeling of the airway wall. Thus, as the disease becomes more severe, the airway, similar to a chronic wound, is more susceptible to a wide range of environmental insults (for example, biologically active allergens, viruses, air pollutants, certain drugs and chemicals), and has an altered repair response with secretion of growth factors that induce mucus cell metaplasia, smooth muscle proliferation, angiogenesis, fibrosis and nerve proliferation. Varying combinations of these processes help explain the different asthma subphenotypes, their response to treatment and their natural history over a person's lifetime.

Most asthma begins in childhood in association with sensitization of the airways to common aeroallergens, especially those derived from house dust mites, cockroaches, animal dander, fungi and pollens. In most cases, this occurs through the selective expansion of T lymphocytes (particularly of the $T_H2$ type) that secrete a cluster of cytokines encoded on chromosome 5q31–33, including interleukins IL-3, IL-4, IL-5, IL-9, IL-13 and granulocyte macrophage colony–stimulating factor (GM-CSF), which are coordinately regulated[1]. Activation of $T_H2$ CD4$^+$ cells occurs through phosphorylation of the *trans*-acting T cell–specific transcription factor GATA-3 (ref. 2). In other affected individuals, alternative immunological pathways drive the inflammatory response. These will be discussed later in this Review.

$T_H2$-type cytokines orchestrate the allergic inflammatory cascade that occurs in asthma, including $T_H2$ cell survival (regulated by IL-4), B cell isotype switching to IgE synthesis (IL-4 and IL-13), mast-cell differentiation and maturation (IL-3, IL-9 and IL-13), eosinophil maturation and survival (IL-3, IL-5 and GM-CSF) and basophil recruitment (IL-3 and GM-CSF). A similar inflammatory response is triggered in response to parasitic infection, suggesting that, in some way, allergy is a manifestation of 'frustrated' parasite elimination.

## Changing worldwide trends in asthma

Asthma and related allergic diseases have increased markedly in Western countries over the past 50 years, and this has been attributed to urbanization and associated changes to diet and lifestyle[3]. The urban-rural gradient in prevalence has been demonstrated most strongly in children who grow up in environments with a wide range of microbial exposures (such as traditional livestock farms or in families who have adopted a more 'naturalistic' diet and lifestyle), who are protected from childhood asthma and atopy (the predisposition to develop IgE against common environmental allergens) in proportion to their level of exposure to bacterial and fungal microbes[4]. This protective effect against the onset of asthma in children is even more apparent if the microbial exposure (for example, working with animals or drinking unpasteurized milk)[5] occurred throughout the mother's pregnancy[6]. To gain further insight into the geographical

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differences in asthma and allergy prevalence, an understanding of the mechanisms underlying the process of allergen sensitization itself is fundamental.

## Allergen sensitization

In association with the airway epithelium and underling mucosa is a specialized population of antigen-presenting (and allergen-presenting) cells (APCs) called dendritic cells (DCs).These cells express receptors of the innate immune system and also have the potential to take up allergens, process them into small peptides and then present them via the major histocompatibility complexes MHC class I and MHC class II for recognition by T cell receptors (**Fig. 1**). Uptake of allergens by airway DCs is an active process; in allergic individuals, it is facilitated by interaction of the allergen with IgE attached to FcεRI, the high-affinity receptor for IgE[7]. At birth, the airways contain no DCs[8]. Damage to and/or activation of the respiratory epithelium by microbes and irritants are probably the main stimuli that initiate the ingression of immature DCs from the bone marrow[9]. These stimuli cause the release of a range of chemoattractants, such as CCL20, CCL19 and CCL27, the ligands for CCR6, CCR7 and CCR10, respectively, which direct DC migration toward the epithelium and underlying mucosa[10] (**Fig. 1**). The presence of GM-CSF, which is released from epithelial cells and a range of other structural and immune cells in the presence of IL-4 and tumor necrosis factor-α (TNF-α), leads to DC maturation to a fully competent, antigen-processing cell. However, during initial allergen sensitization of the airways, the mechanisms responsible for $T_H2$ lymphocyte differentiation from naive T cells remain poorly understood. What is clear is that $T_H2$ polarization requires an obligatory source of IL-4 to activate the transcription factors signal transducer and activator of transcription 6 (STAT6) and GATA-binding protein 3 (GATA3).

Two competing ideas have been advanced to explain the cellular source of the IL-4. First, a $T_H2$ response occurs by default in the absence of strong $T_H1$- or $T_H17$-promoting cytokines or when only a weak MHC class II T cell receptor (TCR) interaction occurs in the immunological synapse, resulting in IL-4 production from naive $CD4^+$ T cells[10]. Alternatively, the obligatory IL-4 may originate from accessory cells, especially mast cells and basophils[11]. Support for this second mechanism comes from the demonstration that, in parasite-infected mice, basophils migrate into the lymph nodes, draining the site of antigen exposure. Here, the basophils serve both as APCs and as the source of IL-4 (ref. 12). Basophil depletion and reconstitution experiments in mice have added credence to this second proposed mechanism. However, the basophil-depleting antibody (antibody to FcεRI) also depletes a subpopulation of 'inflammatory' DCs that express this receptor[10]. Inflammatory DCs, but not basophils, have been suggested to be necessary and sufficient for the development of $T_H2$ immunity to house dust mite allergen when the first exposure occurs by inhalation. For inhaled allergens, such as those from house dust mites, it is proposed that basophils amplify the $T_H2$ immunity that is initiated by DCs and, in part, influenced by innate signaling through Toll-like receptor 4 (TLR4) and C-type lectin signaling on epithelial cells and DCs[13].

Polarization toward a $T_H2$ subtype is also under epigenetic regulation. In mice, microRNA miR-21 has been shown to exert a pivotal role in setting a balance between $T_H1$ and $T_H2$ responses. It does this by binding the promoter of the gene encoding IL-12 p35 and inhibiting its activation in favor of a $T_H2$ profile. Conversely, reduced microRNA levels lead DCs to produce more IL-12, and allergen-challenged T cells to produce more interferon-γ (IFN-γ) and less IL-4, enhancing $T_H1$ delayed–type hypersensitivity[14].

## Propagation of the allergic response

Enhancement of airways sensitization is augmented by two additional factors:

**Breakdown of barrier function.** In developed countries, atopy afflicts up to 40% of children and young adults, but only one-third of cases develop into asthma. Furthermore, systemic atopy itself does not seem to be as important as local sensitization of the lower airways to aeroallergens associated with the development of asthma[15]. Indeed, genome-wide association studies (GWASs) are revealing new asthma susceptibility genes that are mostly expressed in the epithelium and innate immune pathways, including *ORMDL3*, *IL33* and *SMAD3*

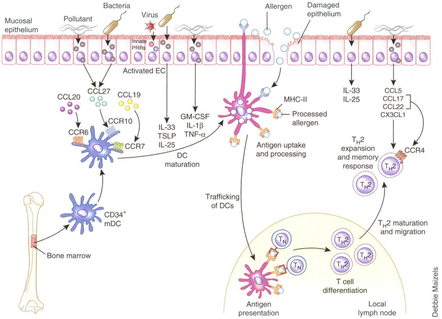


**Figure 1** Primary sensitization of the airways in the induction of allergic-type asthma. Perturbation of the epithelium with infection and pollutants provides the initial danger signal and activates innate signaling receptors, leading to chemokine secretion from airway epithelial cells and trafficking of immature DCs to the mucosal epithelium. DCs respond to danger signals via PRRs, resulting in their maturation to competent antigen-presenting myeloid-type DCs. Once activated, mature DCs process allergens detected through processes extended into the airways, or by capturing allergens that have breached the epithelium. The allergen-loaded DCs then migrate to local lymph nodes where they interact with naive T cells via TCR, MHC class II and co-stimulatory molecules to drive differentiation of T cells. Additional epithelial-derived cytokines and chemokines such as IL-33, IL-25, CCL17 and CCL22 influence DC activation and $T_H2$ maturation and migration into the mucosa.

(ref. 16), rather than genes associated with atopy and serum IgE such as the HLA-DQ locus[16], *FCER1A*, *STAT6* and *IL13* (ref. 17).

The asthmatic epithelium is intrinsically defective in its physical barrier function with incomplete formation of tight junctions, thereby facilitating penetration of inhaled allergens into the airway tissue[18] (**Fig. 1**). Related to this defect, a proportion of the asthma-related allergens have intrinsic biological properties that increase their capacity to penetrate the epithelial barrier and trigger a danger signal to DCs. Components of house dust mite, cockroach, animal and fungal allergens are proteolytic and can disrupt epithelial tight junctions and activate protease-activated receptors[19]. Other allergens have more subtle biological effects (**Table 1**). Beyond proteolytic allergens, additional environmental stimuli such as respiratory viruses and air pollutants (for example, ozone, particulates and environmental tobacco smoke)[20,21] also disrupt tight junctions to impair barrier function.

Defective epithelial barrier function has also been described in the pathophysiology of food allergy[22], allergic rhinosinusitis[23] and conjunctivitis[24]. However, it is in the pathogenesis of atopic dermatitis (or eczema) that genetic defects in barrier function are most clearly understood, with defective filaggrin (FLG) production being the most frequent[25]. In atopic children, it is not infrequent that atopic dermatitis precedes asthma in the 'atopic march', suggesting that epicutaneous allergen transfer may contribute toward the development of asthma[26].

**Enhancement of DC antigen-presenting capacity.** Once sensitized, memory T cells drive the ongoing allergic response through interactions with DCs. DCs extend their processes between epithelial cells into the airway lumen[27], and, by forming tight junctions with adjacent epithelial cells, they can detect inhaled allergenic molecules without disturbing the epithelial barrier[28]. In the mouse lung, two distinct DC subsets have been described in accordance with their expression of the integrin CD11c as myeloid (conventional DCs (cDCs), CD11c$^+$) or plasmacytoid DCs (pDCs, CD11c$^-$)[29]. Similarly, human DCs are subdivided into CD11c$^-$ (CD123$^+$CD303$^+$CD304$^+$) plasmacytoid DCs and CD11c$^+$ myeloid DCs. The myeloid DC can be further subdivided into three subsets on the basis of differential expression of CD1c, CD141 and CD16 (ref. 30). Induced sputum from asthmatic airways and peripheral blood contain increased numbers of both pDCs and cDCs, which further increase in number upon allergen challenge[31].

Proteolytic and other biological activities of allergens (**Table 1**) provide one set of danger signals (PAMPs) to initiate maturation of DCs. Within a few hours of contact with allergen, activation of pattern-recognition receptors (including TLRs on DCs) augments their homing capacity by upregulating chemokine receptors to direct them toward the T cell area of regional lymph nodes[32] (**Fig. 1**). It is the myeloid-type cDC subtypes that are predominantly responsible for antigen presentation, whereas plasmacytoid pDCs are more strongly linked to acquisition of tolerance and antiviral defense. During this time, DCs undergo a substantial phenotypic change, from quiescent cells to cells expressing an array of cell-adhesion and co-stimulatory molecules that are recognized by naive T cells and by the processing of the allergen. Mature DCs occupying the T cell area form an 'immunological synapse' with the few allergen-specific T lymphocytes to initiate a T$_H$ response. Whereas some of the T$_H$ cells make their way to the B-cell follicle to facilitate immunoglobulin class switching from IgM to IgE, others move back to the airway mucosa to elicit the classical T$_H$2 response through the coordinated secretion of the proallergic cytokines.

**Epithelial consolidation of DC activation.** Pattern-recognition receptors (PRRs) have a crucial adjuvant role in directing allergen sensitization. TLRs are key components of the innate immune system that mediate recognition and response to PAMPs in the form of microbial, fungal and viral products and their ligands, including endotoxin (recognized by TLR4), lipoproteins (TLR2 and TLR6), viral double- and single-stranded RNA (TLR3 and TLR7/8) and bacterial CpG-containing DNA (TLR9)[33]. Other PRRs respond to endogenously generated danger signals (DAMPs) produced during tissue damage, such as free ATP and uric acid[34]. Small amounts of TLR ligands from microbes in house dust, when inhaled along with allergens, provide the necessary adjuvant effect to drive the allergic response. Activation of selective TLRs on epithelial cells enhances DC motility and antigen sampling through the production of T$_H$2-promoting chemokines (CCL17 and CCL22 via CCR4)[35] and cytokines (IL-25, IL-33, GM-CSF and thymic stromal lymphopoietin (TSLP)) (**Fig. 1**). In the absence of TLR ligation, however, DCs remain relatively inactive[36].

**Table 1 Examples of biological activities of aeroallergens**

| Source of allergens | Identified biological activities |
|---|---|
| Grass pollen | Pectate lyase, RNase, polygalactouronase, lipid transfer protein, profilin, expansin |
| Tree pollen | Profilin, isoflavone reductase, pathogenesis-related protein, pectin methylesterase, peptidyl-prolyl isomerase, 1,3-β-glucanase, calcium-binding protein, pectate lyase, superoxide dismutase |
| Fungi | Protein disulfide isomerase, aldehyde dehydrogenase, RNase, vacuolar serine protease, alkaline serine protease, enolase, aspartate protease, dipeptidyl peptidase, subtilisin-like protease |
| Pet dander | Uteroglobin-like protein, cystatin, lipocalin, albumin |
| Mites | Cysteine protease, β-glucan moiety, trypsin, amylase, chymotrypsin, chitinase, collagenase, glutathione transferase |

**Viral infection and predisposition to asthma.** The discovery that early-life sensitization to multiple aeroallergens carries the greatest identifiable risk for developing asthma[15] raises the question of what factors result in a predisposition to this phenotype. Although infection with rhinoviruses is the main cause of asthma exacerbation, in those genetically at risk of asthma, rhinovirus-induced wheezing in the first three years of life is also the greatest risk factor for developing asthma at 6 years of age[37]. The range and magnitude of allergen sensitization is also affected by rhinovirus infection[38], with the latter now known to precede the former[39]. As an intrinsic abnormality, impaired TLR3-mediated interferon-β (IFN-β)[40] and IFN-λ[41] production by asthmatic epithelial cells would predispose to both viral lung infection and allergic sensitization[42,43]. Reduced primary IFN production by lower-airway epithelial cells enables rhinovirus and other viruses to replicate, leading to cytotoxic cell death (rather than apoptosis), release of inflammatory products and enhanced viral shedding. Such events provide a strong stimulus for recruitment of immature DCs and their priming for allergen sensitization[9,44]. Asthmatic epithelial cells also respond to rhinovirus infection by generating increased amounts of the pro-T$_H$2 cytokine TSLP[45] (**Fig. 1**), whereas exogenous IFN-β applied to asthmatic epithelium exerts anti-T$_H$2 as well as antiviral properties[46].

## Environmental protection against T$_H$2-mediated sensitization

The ability of microbial and other environmental stimuli to shape the immune response provides a mechanism for the rising trends of asthma in those countries adopting a Western lifestyle and also helps explain protection afforded by traditional livestock-farming and rural lifestyles[47]. The amount and range of microbial exposure, rather than exposure to specific organisms or their products, is what drives protective immunity[48,49]. Previous studies have found that children living on farms have a wider range of microbial exposures than occurs in the reference groups, and this is suggested to explain the protective effect of a farming environment on asthma development[4–6]. As a function of urbanization, loss of microbial biodiversity in air, soil, water and food, along with the adoption of intensive farming methods and use of biocides, widespread use of antimicrobials and disinfectants, changes in the microbiota and dietary changes, go some way in explaining the rise in asthma and allergy in countries adopting components of the western lifestyle[50]. Experimental support for this concept comes from the increase in $T_H2$ and IgE responses in animals bred in the absence of microbial exposures (gnotobiosis)[51].

The idea that exposure to a wide variety of microorganisms and their products protects against allergy and asthma has been reinforced further by the discovery that the microbiome of the airways in asthma is completely different from that of normal subjects. Contrary to popular opinion, the bronchial tree is not sterile; it possesses at least 2,000 bacterial genomes per square centimeter[52]. Pathogenic Proteobacteria (for example, *Haemophilus* spp.) occur most frequently in asthmatic airways of adults and children as compared to normal controls, whereas Bacteroidetes (for example, *Prevotella* spp.) are more frequent in controls. *Haemophilus*, *Moraxella* and *Neisseria* spp. found in neonatal throat swabs have been reported to correlate with an increased risk for asthma in early life, when the disease most frequently begins[53].

## Mechanisms of microbial protection against asthma and allergy

A new paradigm for allergy states that, in genetically susceptible subjects, low-dose exposure to PAMPs and DAMPs in the presence of allergens leads to priming of DCs and enhanced allergic responsiveness[54]. In contrast, high-dose exposure (for example, as occurs on livestock farms, in rural environments in developing countries and in traditional lifestyles) leads to allergenic tolerance[55]. Scalable triggering of innate immunity, as a principal determinant of allergy, is attractive: in different environments, ambient air concentration of the TLR4 agonist, lipopolysaccharide, can vary by as many as 5 logs, with the highest levels being encountered in association with livestock farming[4] and pet ownership[56].

**Immunological tolerance in protecting against asthma.** Dampening of immunological responsiveness and induction of allergen tolerance have been attributed to the regulatory T ($T_{reg}$) cell. $T_{reg}$ cells are identifiable through their coexpression of the transcription factor forkhead box protein 3 (FoxpP3), CD4 and CD25, and they exist in either naturally occurring (thymus-derived or $nT_{reg}$) or inducible ($T_R1$ or $iT_{reg}$) forms[57]. The redirection of allergen-specific effector T cells to a regulatory phenotype is a key event in the development of a normal immune response to allergens, and it accounts for at least some of the clinical efficacy of allergen-specific immunotherapy. Naturally occurring $T_{reg}$ cells influence allergic pathways by secreting IL-10 and TGF-β to mediate (i) suppression of DCs involved in programming effector T cells, (ii) direct inhibition of $T_H1$, $T_H2$ and $T_H17$ cells, (iii) suppression of allergen-specific IgE and induction of IgG4, (iv) inhibition of mast cells, basophils and eosinophils and (v) prevention of effector T cell migration into the target tissue[58] (**Fig. 2**). Although controversy remains over how IgG4 is able to suppress allergic responses, this process probably involves activation of immunoreceptor tyrosine-based inhibitory motif (ITIM) signaling through aggregation of the low-affinity IgG receptors FcgRIIB and FcgRIIA on mast cells and basophils, rather than inhibiting allergen binding to IgE[59].

**Early-life protection against allergy and asthma.** Prenatal exposure to high levels of microbial products (for example, in milking parlors and cow sheds) is especially protective, and although multiple mechanisms have been suggested, the inhibitory response seems most closely linked to high circulating numbers of $T_{reg}$ cells[60]. In mice, selective depletion of $T_{reg}$ cells during the allergen-sensitization phase markedly enhances experimental allergic lung inflammation[61]. A study in humans showed that maternal cells have the capacity to traverse the placenta and enter fetal lymph nodes to induce $iT_{reg}$ cells, preventing antimaternal immunity that persists until early adulthood. This study also challenges the idea that fetal lymphocytes are incapable of mounting an immune response[62]. A similar mechanism of maternal-fetal transfer may operate in the protection against allergic sensitization to induce allergen-specific $T_{reg}$ cells in the presence of TGF-β. However, although membrane-bound TGF-β has been implicated in mucosal tolerance to microorganisms[63], answering the question of how these pathways are activated in the presence of high maternal and offspring microbial exposure involving PRRs and epigenetic pathways requires further research.

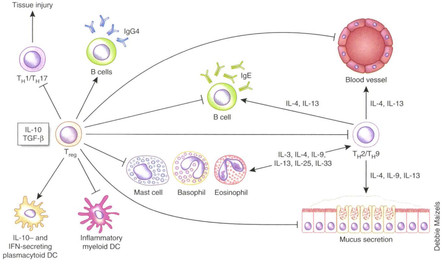
Further strong support for maternal programming of protection against infant sensitization comes from exposing pregnant mice to the Gram-negative, nonpathogenic bacterium *Acinetobacter lwoffii* F78, which is found in cow sheds and activates TLR2, TLR3, TLR4, TLR7 and TLR9. Prenatal exposure of pregnant mice to *A. lwoffii* F78 protects the offspring from developing $T_H2$-mediated allergic airway inflammation[64]. The mechanisms resulting in this protection involve the upregulation of maternal lung TLR expression, initiation of a transient, acute, local $T_H1$-like inflammatory response, systemic release of inflammatory cytokines and downregulation of TLR and cytokine expression in the placenta. Offspring from *A. lwoffii* F78–treated *TLR2/3/4/7/9*$^{-/-}$ mothers were no longer protected from $T_H2$-type airway responses to inhaled antigen; thus, maternal TLR signaling seems to be crucial in mediating the transfer of this transgenerational protective effect. The inhibitory effects of *A. lwoffii* F78 on allergic responsiveness in the offspring is dependent on IFN-γ and epigenetically controlled through chromatin histone 4 (H4) acetylation of the *IFNG* promoter of $CD4^+$ T cells; conversely, inhibition of H4 acetylation abolishes the $T_H2$-protective phenotype[65].

## Engagement of adaptive cellular immunity in asthma

Once DCs have become primed by the environment to promote allergen sensitization, a common sequence of events occurs. Presentation of allergen peptides in the cleft of MHC class II to the TCR on naive T cells in parallel with engagement of co-stimulatory molecules on each cell type (for example, CD80 or CD86 on DCs with CD28 on T cells, or OX40L on DCs with OX40 on T cells) in the presence of low levels of IL-12 and high levels of IL-4 results in T-cell differentiation toward the $T_H2$ phenotype (**Fig. 1**). If the co-stimulatory second signal in the immunological synapse is missing (for example, engagement of CD80/86 with cytotoxic T-lymphocyte antigen-4 (CTLA-4) on T cells), then anergy or T cell apoptosis results[66].

Among the family of co-stimulatory molecules, OX40L is selectively upregulated by the IL-7-like cytokine TSLP, which derives largely from the epithelium following activation of TLRs[67]. Several other newly identified epithelial-derived cytokines have also been shown to exert $T_H2$-polarizing activity. These include the IL-1 and

**Figure 2** The pleiotropic effects of T$_\mathrm{reg}$ cells in suppressing allergic inflammatory pathways. T$_\mathrm{reg}$ cell development and suppression is fundamental in protecting normal airways against allergen sensitization. T$_\mathrm{reg}$ cells are also induced in response to allergen-specific immunotherapy and promote the production of the 'blocking' antibody class IgG4. Solid arrows indicate stimulatory signals; blunt arrows indicate inhibitory signals.



IL-18 family member IL-33 acting through its receptor, ST2 (ref. 68), and IL-25, one of six members of the IL-17 cytokine family[69]. TSLP, IL-33 and IL-25 are generated by the airway epithelium in response to activation of PRRs such as TLR3 (and its ligand, viral double-stranded RNA) and TLR5 (and its ligand, bacterial flagellin) or following cytotoxic epithelial injury. IL-33 functions as an alarmin to alert the immune system to physical stress or infection (**Fig. 1**). The IL-33 receptor, comprising ST2 and IL-1 receptor accessory protein, is expressed at a high level on T cells and mast cells, where its activation expands the T$_\mathrm{H}$2 cell population (possibly via induction of nuocytes) and greatly enhances release of their cytokines[68]. In contrast, IL-25 functions to enhance the T$_\mathrm{H}$2 memory response initiated by TSLP-primed DCs[69], with marked upregulation of IL-25 and its receptor occurring in asthmatic airways following allergen challenge[70]. All three cytokines also interact with effector cells of the allergic response—mast cells, basophils, eosinophils and endothelial cells—to enhance their inflammatory functions[71]. Thus, by operating together, these three epithelial cytokines have the potential to bridge innate and adaptive immunity to sustain the T$_\mathrm{H}$2 response toward a more chronic state that is characteristic of asthma.

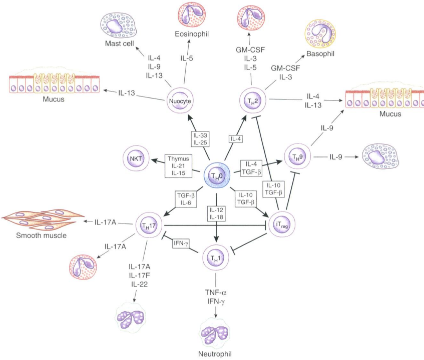
The recent recognition of a new, innate type-2 immune effector leukocyte, the nuocyte, is an exciting new breakthrough in allergic disease in providing a missing link between the innate and adaptive T$_\mathrm{H}$2 response (**Fig. 3**). Nuocytes expand in number in response to epithelial-derived IL-25, IL-33 (similar to IL-12 in driving the T$_\mathrm{H}$1 response)[72], Notch signaling and activation of the transcription factor retinoic acid–related orphan receptor γt (RORγt) and are an early source of IL-13, as shown in gastrointestinal elimination of parasites. Thus, in the absence of both IL-25 and IL-33, nuocytes fail to expand, resulting in defective worm expulsion, which is reversible by the adoptive transfer of *in vitro*–cultured wild-type, but not IL-13–deficient, nuocytes[73]. Thus, nuocytes represent a new, key innate effector cell in type-2 immunity, and recent observations suggest that this cell population is also incriminated in allergic airway sensitization and parasite-induced lung eosinophilia in mouse models[74,75]. It is now crucial to show whether nuocytes have a role in the pathophysiology of asthma in humans.

### New T cell subsets in asthma subphenotypes

**T$_\mathrm{H}$9 cells.** A second T$_\mathrm{H}$2 cell subtype, designated T$_\mathrm{H}$9, has been linked to asthma as a subpopulation that requires both IL-4 and TGF-β[76] (**Fig. 3**). During the allergic response *in vivo*, it remains to be established whether IL-9–secreting T cells are distinct from T$_\mathrm{H}$2 cells or whether T$_\mathrm{H}$2 cells become reprogrammed into T$_\mathrm{H}$9 cells. T$_\mathrm{H}$9 cells do not express well-defined transcription factors such as T-bet, GATA3, RORγt and Foxp3, indicating that they are different from T$_\mathrm{H}$1, T$_\mathrm{H}$17 and iT$_\mathrm{reg}$ cells. The sequential cytokine interactions that lead to T$_\mathrm{H}$9 differentiation are becoming clearer[77]. TGF-β induces the PU.1-encoding *Sfpi1* locus, which is independent of IL-4–induced STAT6 activation, whereas IL-4 represses T-bet and Foxp3 (inhibitors of IL-9 production) but is required for the induction of T$_\mathrm{H}$9-promoting IRF4 (ref. 78).

Signaling through IL-2R and CD28 can increase IL-9 secretion by allergen-specific T cells and requires the simultaneous presence of both IL-4 and IL-10, although IL-4–independent production of IL-9 has also been demonstrated from human memory (CD45RO$^+$ CCR8$^+$) T cells, nT$_\mathrm{reg}$ cells in peripheral tissues and iT$_\mathrm{reg}$ cells in the presence of TGF-β. Other sources of IL-9 relevant to diseases such as asthma include eosinophils, basophils and mast cells. IL-9 is highly expressed and localized to tissue lymphocytes during intestinal parasite infection[79] and to CD3$^+$ cells in bronchial submucosa and bronchoalveolar lavage[80]. IL-9 expression also increases markedly in response to allergen challenge[81]. In studies using IL-9 transgenic and knockout mice, direct IL-9 instillation into the lungs and blocking monoclonal antibodies (mAbs), it has been shown that IL-9 drives mucus production, both by a direct effect on airway epithelia[82] and also by interacting with IL-13 (ref. 83). Other features of airway remodeling such as subepithelial fibrosis have also been described in IL-9 transgenic mice, possibly owing to immune-cell recruitment (for example, by promoting eotaxin release from smooth muscle) and inflammatory-mediator release[84]. Along with IL-4 and stem cell factor, IL-9 is also a potent stimulus for mast-cell development[84]. As IL-9 has been implicated in both inflammatory and remodeling components in mouse models of allergic airway disease, it seems an attractive therapeutic target. Two first-in-human, open-label dose-escalation trials of a monoclonal antibody against IL-9, MEDI-528, in normal subjects and subjects with mild asthma have been successfully completed, showing some evidence of efficacy[85].

**T$_\mathrm{H}$17 cells.** T$_\mathrm{H}$17 cells are a distinct T cell lineage that does not share developmental pathways with either T$_\mathrm{H}$1 or T$_\mathrm{H}$2 cells. Indeed, both IFN-γ and IL-4 inhibit IL-17–producing T cells, whereas blocking antibodies to IFN-γ and IL-4 are facilitatory[86,87]. T$_\mathrm{H}$17 cells have a role in regulating both neutrophilic and macrophage inflammation in autoimmune disease and psoriasis, and more recently they have been suggested to be involved in asthma and corticosteroid insensitivity[88]. T$_\mathrm{H}$17 lymphocytes differentiate in response to a combination of TGF-β and IL-6, leading to the expression of the



Debbie Maizels

**Figure 3** Different T cell subtypes involved in the pathogenesis of asthma and its differing endotypes. Combinations of cytokines and contact signals cause DCs and the thymic epithelium to conspire to drive the differentiation of an array of different leukocyte subsets (inner, blue circle) that augment or protect the airways from inflammatory responses linked to asthma. These different leukocyte subsets generate cytokines that influence different cell types and the attendant inflammatory response, driving allergic airway inflammation and airway hyperreactivity.

transcription factor RORγt. Conversely, their differentiation is restricted by both $T_{H}1$ and $T_{H}2$ cytokines including IFN-γ, IL-4 and IL-13 (ref. 87). $T_{H}17$ cells are characterized by the production of IL-17A, IL-17F and IL-22. IL-17A and IL-17F both induce IL-6, GM-CSF, CXCL10 and CXCL8 in epithelial and endothelial cells, fibroblasts, neutrophils and eosinophils[89]. Specifically, the induction of CXCL8, a potent neutrophil chemokine whose expression is elevated in airway secretions in severe asthma, has directly implicated $T_{H}17$ cells in neutrophilic airway inflammation[90].

In mice, allergic sensitization followed by challenge of the airways induces a strong $T_{H}17$ response in association with airway neutrophilia and hyperresponsiveness, and this response is abrogated in *Il17f*$^{-/-}$ mice[91]. Thus, although IL-17 is considered to be essential during antigen sensitization to establish allergic airway responses, in mice already sensitized to allergen, IL-17 attenuates the allergic response by inhibiting DCs and chemokine (CCL11 and CCL17) synthesis[92]. Also in mice, there seems to be an inverse relationship between IL-25 and IL-17A in regulating allergic lung responses. In ovalbumin-driven allergic-type lung inflammation, neutralization of IL-25 correlates with a decrease in IL-13 concentrations, but an increase in IL-17A production, and prevention of AHR. In contrast, blockade of IL-17A reverses any protective effect of antibodies to IL-25 including re-expression of IL-13 and eotaxin production, eosinophilia and AHR[93]. Moreover, IL-17A itself, but not IL-17F or IL-22, enhances the contractile force of airway smooth muscle. Sensitized mice lacking the integrin $\alpha_v\beta_8$ on DCs show reduced activation of this IL-17A–linked pathway with antigen challenge. This reduction in smooth muscle contraction in the airways is reversible by IL-17A, indicating involvement of this cytokine on allergen-induced AHR by acting directly on airway smooth muscle[94].

Experimental models of allergic rhinitis and asthma have also suggested that the complement cleavage products C3a and C5a, and activation of their corresponding receptors in antigen-presenting cells, regulate the development of altered $T_{H}2$ and $T_{H}17$ immunity[95]. Thus, C3aR-deficient mice have impaired $T_{H}2$ responses, along with protection from AHR, following allergic antigenic stimulation[96,97]. Similarly, when C5a is inhibited, the severity of AHR is reduced during the effector phase of the allergic airway response in rodents[98]. Complement probably has a multifunctional role in the allergic tissue response: on the one hand, it exacerbates AHR and the $T_{H}2$ response during the effector phase; on the other, it induces tolerance during allergen presentation to T cells[99].

In humans, a newly recognized subset of $T_{H}2$ memory and effector cells has been found expressing both GATA3 and RORγt and, as a consequence, producing $T_{H}17$ and $T_{H}2$ cytokines[100]. However, although a good case can be made for IL-17A and IL-17F in mouse models of neutrophilic and corticosteroid-refractory lung responses to allergens, evidence for IL-17 involvement in human asthma is less robust, despite some emerging genetic evidence and a potential role for IL-17A and IL-17F in moderate-to-severe disease[101,102].

Several groups have reported that the number of circulating $T_{H}17$ cells as well as plasma concentrations of IL-17 and IL-22 increase in proportion to disease severity. However, in a recent bronchial biopsy in asthma of varying severity compared to normal controls, the main cellular source of IL-17 was neutrophils, not T cells, with no correlation between IL-17A or IL-17F expression and the extent of neutrophilia, nor any link to asthma severity[102]. Rather, the number of bronchial submucosal IL-17F$^+$ cells correlated most closely with the extent of eosinophilia. It is clear that functional evidence is needed to associate $T_{H}17$ cells causally to a specific subphenotype of human asthma. It should be noted that prosurvival factors for neutrophils are also produced by the epithelium in severe asthma and asthma associated with current tobacco smoking[103], possibly involving transactivation of the epidermal growth factor receptor[104].

**Invariant natural killer T cells.** Invariant natural killer T (iNKT) cells are characterized by an invariant variable region TCR α-chain 14–joining region 18 (V(α)14-J(α)18), which selectively recognizes the glycolipid α-galactosylceramide (α-GalCer) presented to the TCR by the antigen-presenting molecule CD1d[105]. Recently, the endogenous lipid β-D-glucopyranosylceramide (β-GlcCer) has been shown to be a potent cell self antigen in mice and humans, and its activity is dependent upon the composition of the N-acyl chain. Because β-GlcCer is generated in response to TLR agonists, it is suggested that recognition of β-GlcCer by CD1d translates innate danger signals into iNKT cell activation[106].

A role for subsets of iNKT cells in asthma has also been suggested by extensive studies in animal (mostly mouse) models of lung inflammation driven by allergen, viral infection, ozone exposure or bacterial components. This idea requires that, in causing airway hyperreactivity, iNKT cells function together with $T_H2$ cells or independently of adaptive immunity[107]. However, whereas the mouse studies are highly suggestive, the role of CD1d-restricted iNKT cells in human asthma remains controversial. An initial study reporting that iNKT cells were the predominant T cells in the lung and bronchoalveolar lavage fluid of all asthmatic subjects studied[108] has not subsequently been confirmed. Indeed, the methods used to detect human iNKT cells in this first report were suboptimal. Instead, iNKT cells seem to be present only as a small fraction of the total T cells populating asthmatic airways[109]. An emerging view is that iNKT cells are likely to have a role in modulating the asthmatic phenotype, but they are probably not the critical drivers of the asthmatic response, as originally suggested. However, in line with a modifying influence, a recent study reported that most sterile house dust extracts contain antigens capable of activating both mouse and human iNKT cells[110].

## The humoral immune response

Isotype switching of B cells to IgE synthesis is a prerequisite of atopy and provides the critical trigger mechanism for the immediate allergic response upon exposure to allergen. This process requires the obligatory presence of IL-4 or IL-13, which, by interacting with its receptor on B cells in the presence of MHC class II–associated allergen presentation by $T_H2$ cells and co-stimulation involving CD40 and CD40L, results in immunoglobulin class switching from IgM to IgE. The intracellular molecular sequence involves germline gene transcription, DNA recombination and B cell differentiation, resulting in the synthesis and secretion of allergen-specific IgE antibody, followed by the maturation of clonal B cell subsets into plasma cells secreting large amounts of IgE[111]. IgE antibodies with high affinity for allergens undergo cross-linking at low concentrations by trace amounts of allergen, whereas IgE antibodies with low affinity bind allergens weakly. High-affinity IgE is formed by sequential class switching ($\mu \rightarrow \gamma \rightarrow \varepsilon$), with an intermediary IgG phase required for the affinity maturation of the IgE response[112]. As a result, the IgE inherits somatic hypermutations and high affinity from the IgG1 phase. Low-affinity IgE is formed through direct class switching ($\mu \rightarrow \varepsilon$) and is therefore much less mutated. It follows that mice deficient in IgG1 production cannot produce high-affinity IgE, even with repeated immunizations. Low-affinity IgE competes with high-affinity IgE for binding to Fcε receptors, thereby reducing IgE-dependent mast-cell and basophil activation-secretion coupling[112].

IgE binds the α-chain of high-affinity FcεR1 on mast cells and basophils. Cross-linking of receptor-bound IgE by allergen initiates cell activation and the release of preformed and newly generated mediators, cytokines, chemokines and growth factors. FcεR1 receptors facilitate allergen uptake and processing, with the extent of IgE occupancy of FcεRI positively regulating the level of receptor expression. IgE can also bind low-affinity FcεRII (CD23) on B cells, where it serves to enhance both allergen-specific B cell and T cell recall functions[113]. In the bronchial mucosa of asthmatics, class switching to IgE has been shown to occur irrespective of atopic status[114], providing a possible mechanism for non-atopic as well as atopic asthma. More than 50% of individuals with non-atopic asthma also have circulating IgE antibody against *Staphylococcus aureus* endotoxins, which serve as superantigens by interacting with selective TCR Vβ families[115].

## Modulation of innate and adaptive immunity in asthma

The sentinel role of T cells, especially of the $T_H2$ type, in orchestrating the inflammatory response in asthma makes them a particularly attractive therapeutic target.

**Immunosuppressants in difficult asthma.** Severe corticosteroid 'refractory' asthma presents a particularly difficult problem, as it is associated with the greatest morbidity and mortality and the accompanying health service and economic costs. Many attempts to treat this condition have used immunosuppressants such as gold salts, methotrexate, azathioprine, cyclosporine and tacrolimus, but, although some clinical benefits have been found in individual patients, the beneficial effects are sporadic and often associated with unacceptable side effects. Intravenous immunoglobulin also has limited use in severe asthma, by reducing T cell activation and increasing corticosteroid responsiveness[116]. A more recent approach has been the use of biologics to selectively block T cell responses. Keliximab, a chimeric mAb targeted to the CD4 receptor, when administered to individuals with moderate-to-severe corticosteroid-dependent asthma produced some beneficial effect on the diurnal variation in lung function. However, it had limited effect on other asthma end points[117], despite markedly reducing T cell numbers, CD4 receptor density and allergen-driven T cell proliferation[118].

In asthmatic airways and circulating cells, the extent of cell-surface CD25 expression increases in proportion to disease severity. Daclizumab is a blocking humanized mAb to CD25 and a powerful immunosuppressant used in the management of organ transplant rejection[119]. In moderate-to-severe asthma, treatment with daclizumab for 150 d resulted in significant but small improvements in asthma outcome measures but was accompanied by the expected side effects of immunosuppression[120].

Following allergen challenge in asthma, $T_H2$ cells are recruited into the airways by the release of CCL17 and CCL22 from epithelial cells interacting with CCR4 on $T_H2$ cells[121]. The supernatant from allergen-challenged asthmatic bronchial explants is highly chemoattractant to $T_H2$ cells, and its chemoattractant activity is completely blocked by a mAb to CCR4 (ref. 122). A single injection of a defucosylated human IgG mAb to CCR4 with enhanced antibody-dependent cell-mediated cytotoxicity (mogamulizumab, AMG 761) in nonhuman primates and in atopic normal subjects caused profound and sustained reductions in numbers of circulating $T_H2$ cells lasting several months[123]. Amgen is planning a phase 2 trial of mogamulizumab in asthma, but this awaits the selection of doses that are sufficient to attenuate $T_H2$ cytokine production without depleting $T_H2$ cells. Therapeutic efficacy of mogamulizumab has already been shown in mycosis fungoides, Sézary's syndrome and related T cell lymphomas involving CCR4-bearing T cells[124].

Another $T_H2$ chemoattractant is prostaglandin $D_2$ ($PGD_2$), an eicosanoid produced in large amounts from activated mast cells[125]. The G protein–coupled receptors TP1 and DP1 mediate $PGD_2$'s bronchospastic and vasodilator actions, respectively; however, stimulation of the third $PGD_2$ receptor, CRTH2 (DP2), which is expressed on $T_H2$ cells, eosinophils and basophils, leads to their recruitment into the airways, as well as promoting cytokine release[126]. Potent small-molecule CRTH2 antagonists such as MK-7246 are highly active at inhibiting eosinophilic responses in allergen-challenged animal models[127]. However, whereas in asthma CRTH2 expression is restricted to only 2.3% of airway T cells compared to 0.3% in normal airways, a recent 4-week clinical trial of the orally active antagonist OC000459 (Oxigen) has shown some efficacy in moderate disease not requiring corticosteroids, and caused a 60% reduction in the frequency of sputum eosinophils[128].

An attractive approach to attenuating T cell responses in asthma is to block T cell and DC co-stimulation through the 'second signal' (**Fig. 1**). Maturation and activation of T cells involving

MHC-restricted antigen presentation requires parallel engagement of CD28 on T cells by CD80 or CD86 (second signal)[66]. This co-stimulation is interrupted if CD28 interacts with CTLA-4, another adhesion molecule that delivers a suppressive signal to T cells by inhibiting Akt phosphorylation to induce anergy or apoptosis, and directs early T cell differentiation into Foxp3$^+$ $T_{reg}$ cells[129]. A human immunoglobulin CTLA-4 fusion protein (abatacept) has been developed that is efficacious in rheumatoid arthritis[130] and in slowing the loss of pancreatic islet β cells in type 1 diabetes and that is associated with increasing the proportion of $T_{reg}$ cells in insulitic lesions[131]. In human asthmatic bronchial explants, CTLA-4 fusion protein inhibits allergen-specific $T_H2$ cytokine[132] and chemokine[133] release, but only in explants from the airways of mild allergic asthmatics[134]. A trial in mild atopic asthma is investigating whether abatacept is effective in decreasing allergic airway inflammation following segmental allergen challenge (http://clinicaltrials.gov/ct2/show/NCT00784459). A phase 2 trial to evaluate the efficacy, safety and tolerability of an intravenous human mAb to OX40L (RO4989991) in the prevention of allergen-induced asthma has also just been completed, and the outcome is awaited (http://clinicaltrials.gov/ct2/show/NCT00983658).

**Targeting $T_H2$ cytokines in asthma.** The key cytokines incriminated in $T_H2$-type inflammation are those encoded in the IL-4 gene cluster on chromosome 5q31, containing the genes encoding IL-3, IL-4, IL-5, IL-9, IL-13 and GM-CSF[1]. Confidence in the $T_H2$ pathway as being crucial to asthma pathophysiology has been the driving force for a range of biologics targeting their specific cytokines. These have mostly been directed at the IL-4 and IL-13, IL-5 and, for $T_H1$, the TNF pathways. What is clear from all these studies is that overall clinical efficacy is relatively low but, in each of the tested populations, substratification has identified a small, well-defined responder group[135].

A good example is the IL-4 and IL-13 pathway. Although there is overwhelming evidence from *in vitro* and animal (mostly mouse) studies indicating a sentinel role for this cytokine pathway in allergic processes[136], attempts to validate this hypothesis in human asthma revealed that only 50% of individuals with asthma had elevated IL-13 levels in sputum, irrespective of the severity of the disease[137].

To emphasize the importance of disease substratification, Woodruff *et al.*[138] have shown that only 50% of individuals with asthma attending a clinic express IL-13–responsive genes in their airway epithelial cells, and this is linked to a strong $T_H2$ ('$T_H2$-high') response in bronchial biopsies, as opposed to in '$T_H2$-low' asthmatics, whose IL-13–responsive gene expression signature was indistinguishable from that of normal subjects. $T_H2$-high asthmatics had significantly greater expression of IL-5 and IL-13 in bronchial biopsies along with greater AHR and higher serum IgE, blood and airway eosinophilia, subepithelial fibrosis and airway mucin gene expression. The clinical response with a trial of inhaled corticosteroids was also restricted to $T_H2$-high asthmatics only[138]. If only half of the subjects with asthma who had been enrolled into clinical trials to examine the efficacy of IL-4 and IL-13 blockade engaged the IL-13 pathway as a key component of pathobiology, then perhaps the negative outcomes of trials involving unselected patients is to be expected. One IL-13 biomarker in the Woodruff study was periostin[138]. In a recently published trial, the mAb to IL-13, lebrikizumab (Genentech/Roche), when administered to patients with chronic moderate-to-severe asthma for 12 weeks, significantly increased baseline spirometry (5.5%). This result was enhanced in those with elevated serum periostin (high periostin 8.2% versus low periostin 1.2%)[139]. However, as with the other IL-13–specific antibody trials (AMG-307 and CAT-354), there was little or no effect on patient-related outcome measures.

IL-5 is a key cytokine involved in eosinophil maturation and survival whose blockade in various animal models has a strong effect on acute and more sustained pulmonary eosinophilia and attendant changes in lung function. However, despite markedly reducing both circulating and sputum eosinophilia, two humanized mAbs, mepolizumab and reslizumab, when administered to patients with moderate-to-severe asthma, had no overall effect on any asthma outcome measures. Nevertheless, two studies of mepolizumab in patients with severe asthma requiring oral corticosteroids and persistent sputum eosinophilia showed a good clinical response[140,141], as also found in Churg-Strauss and other hypereosinophilic syndromes[142]. Similar results have also been obtained with reslizumab[143,144]. Efficacy of mepolizumab has also been described in severe eosinophilic nasal polyposis in proportion to nasal lavage IL-5 levels[145]. A further development of this approach has been the introduction of a highly active mAb targeting IL-5Rα (MEDI 563, benralizumab), which has been defucosylated to enhance its antibody-dependent cell-mediated cytotoxicity potential[146]. A phase 1 study in mild asthma has shown a strong dose-related reduction of circulating eosinophils lasting 8–12 weeks after a single injection[147].

Targeting TNF-α in severe asthma with golimumab also yields responders and nonresponders[148]. In the case of anti-TNFα therapies, which include the soluble receptor fusion protein etanercept and mAbs, the identification of more neutrophilic asthma that is less dependent upon $T_H2$ mechanisms and, as a consequence, less responsive to corticosteroids might help identify a responsive target subpopulation. Such patients have been shown to have high circulating TNF-α and CXCL-8 as biomarkers[149]. A transcriptomic analysis applied to induced sputum from such patients has identified a unique signature with prominence of IL-1 and the TNF-α and nuclear factor-κB pathways[150]. This stratification of asthma into pathway-selective phenotypes is likely to be a key driver for future drug development, as is proving so successful for cancer treatments.

## BOX 1 Unanswered questions relating to adaptive and innate immunity in allergy and asthma

How does the epithelium program DCs during initial allergen sensitization and upon re-exposure?

What is the relationship between the increase in and altered function of smooth muscle cells and the immune and inflammatory responses in asthma?

What factors localize the immune and inflammatory responses to the conducting airways while sparing the alveoli?

How do the different immune and inflammatory phenotypes relate to different asthma endotypes, their responses to treatment and natural history?

What are the prenatal immune and structural changes in the lungs that predispose someone to acquiring an asthma phenotype?

What roles do biological properties of some allergens have in enhancing sensitization of the airways?

What are the causative mechanisms for the protective effects of microbes and their products in allergy and asthma?

## Concluding comments

Knowledge of the underlying mechanisms of allergen sensitization and the airway events that follow allergen exposure has provided an important underpinning of the cellular and mediator mechanisms of asthma. However, important questions remain to be answered, such as how genetic factors predispose individuals to asthma (beyond atopy), the precise mechanisms for the rising trends in disease associated with the Western lifestyle, how exposure to microorganisms at crucial stages across the life course help to shape the asthmatic response or protect against it, and the cellular mechanisms that connect multiple different environmental exposures to asthma persistence and chronicity (see **Box 1**).

Knowledge gained from detailed immunology *in vitro* and in animal models of allergic-type lung inflammation has transformed our understanding of the crucial roles of both the innate and adaptive immune responses pertinent to asthma. However, what is disappointing is the lack of translation of this mechanistic understanding into patient benefit, unlike what has occurred for some other chronic immunological and inflammatory diseases (for instance, rheumatoid arthritis, systemic lupus erythematosus, psoriasis and inflammatory bowel disease). Part of this lack of success may rest on the reliance on relatively simple antigen sensitization and challenge models as surrogates for human asthma[151]. What are needed are models that more closely reflect asthma pathophysiology as it occurs in humans, and we should harness some of the new 'omics' technologies to explore in detail how different pathways translate into disease subtypes in deeply phenotyped patients. The identification of these pathways and their biomarkers, coupled with targeted biologics, will undoubtedly transform disease management, especially if these are upstream factors in the pathogenetic sequence and closer to the disease origins where stratified medicine is most effective[135]. Moreover, a greater understanding of the early-life origins and factors leading to disease consolidation and persistence will open new doors for both asthma prevention and treatment.

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# The airway epithelium in asthma

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**Asthma is a T lymphocyte–controlled disease of the airway wall caused by inflammation, overproduction of mucus and airway wall remodeling leading to bronchial hyperreactivity and airway obstruction. The airway epithelium is considered an essential controller of inflammatory, immune and regenerative responses to allergens, viruses and environmental pollutants that contribute to asthma pathogenesis. Epithelial cells express pattern recognition receptors that detect environmental stimuli and secrete endogenous danger signals, thereby activating dendritic cells and bridging innate and adaptive immunity. Improved understanding of the epithelium's function in maintaining the integrity of the airways and its dysfunction in asthma has provided important mechanistic insight into how asthma is initiated and perpetuated and could provide a framework by which to select new therapeutic strategies that prevent exacerbations and alter the natural course of the disease.**

## INTRODUCTION

Asthma is a chronic inflammatory airway disorder that leads to symptoms such as coughing, wheezing and chest tightness. Airway obstruction runs a variable course, with symptom-free periods interrupted by periods of exacerbations, often caused by viral infection. A typical feature is bronchial hyperreactivity—the tendency of the airway to constrict in response to stimuli such as cold air and exercise. Allergic sensitization to inhaled allergens such as house dust mites (HDMs), animal dander, pollen or fungal spores is often found in children with asthma, and this is due to atopy, which is a predisposition to develop allergic hypersensitivity reactions and produce IgE in response to allergens. Only half of adults who have asthma are atopic, but the disease otherwise presents similarly in the clinic[1].

In bronchial biopsies or lung-resection samples, asthma is characterized by accumulation of eosinophils, mast cells and $CD4^+$ T lymphocytes producing interleukin-4 (IL-4) and/or IL-5 in the epithelium and lamina propria, even in individuals who have non-atopic asthma[2–4]. Animal models in which $T_H2$-type cytokines such as IL-4, IL-5 or IL-13 have been individually knocked out have provided important evidence that the $T_H2$ axis can drive eosinophilic airway inflammation and bronchial hyperreactivity[5]. A $T_H2$-biased response also seems to be detectable in 50% of individuals with asthma[6]. This finding of distinct clinical subsets of asthma is also reflected in clinical trials employing biologicals aimed at blocking $T_H2$ cytokines, wherein drugs targeting IL-5 are effective, but only in a subset of patients[7]. In some individuals with asthma, particularly those who respond poorly to steroids, airway infiltrates are composed primarily of neutrophils, which are probably recruited to the airways by IL-17–producing cells such as $T_H17$ lymphocytes or $\gamma\delta$T cells[1,8]. Despite these differences in the underlying etiology of disease, in almost all forms of asthma there are structural changes to the airway wall, most often called airway remodeling. These changes consist of an increase in the smooth-muscle mass surrounding the airway wall, a deposition of extracellular matrix components under the epithelial basement membrane that causes a thickened appearance, a breach in the integrity of the airway epithelium and an increase of mucus-producing goblet cells in the epithelium or submucosal glands. Initially, these structural changes were considered a consequence of sustained airway inflammation, akin to a scarring reaction in response to chronic injury. However, recent data have led to a paradigm shift, with the structural components of the lung being shown to respond to environmental risk factors for asthma[9] and to have profound upstream effects in initiating and sustaining the allergic cascade[10].

### Epithelial cells as frontline activators of dendritic cells

Airway epithelial cells (ECs) lie at the interface between the host and the environment and represent the first line of defense against microorganisms, gases and allergens[11]. In their exposed position, ECs express many pattern recognition receptors (PRRs) to rapidly detect and respond to pathogen-associated molecular patterns (PAMPs) found in microbes or to damage-associated molecular patterns (DAMPs) released upon tissue damage, cell death or cellular stress (**Box 1**). The activation of epithelial PRRs leads to the release of cytokines, chemokines and antimicrobial peptides that attract and activate innate and adaptive immune cells (**Fig. 1**). Recent studies have demonstrated that EC activation is a key triggering event in the recognition of inhaled allergens that activates the local network of dendritic cells (DCs), which coordinate the subsequent immune response[12].

DCs are professional antigen-presenting cells of the lung immune system and have the ability to sample the inhaled air for antigens by extending dendritic processes across the epithelial layer while maintaining barrier function by formation of tight junctions[13].

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They subsequently migrate to the draining mediastinal lymph nodes and present antigen to recirculating naive and memory T cells[14] (**Fig. 1**). When lung DCs were isolated from the airways of allergen-exposed mice and transferred to naive mice, they induced allergen-specific $T_{H}2$ cells[15]. Conversely, when DCs were depleted from the airways of naive or sensitized mice, $T_{H}2$ sensitization or development of allergic inflammation in response to allergen exposure did not develop[15,16]. As DCs express most PRRs, it was originally assumed that DCs directly recognize inhaled foreign substances. However, experiments using radiation-chimera mice lacking Toll-like receptor 4 (TLR4) on either radiosensitive hematopoietic or radioresistant structural cells revealed that the recruitment, activation and intraepithelial migration of DCs in response to inhaled endotoxin requires only epithelial TLR4 triggering[17]. When studying the response to inhaled HDM allergen, we similarly found a crucial requirement for epithelial TLR4 expression in promoting recruitment and activation of DCs and subsequent development of allergy to HDM[17], findings that have been extended to other model allergens[18].

## BOX 1 Expression of pattern recognition receptors by lung epithelium

***TLRs.*** Like cells of the innate immune system, respiratory ECs express cell-surface receptors and endosomal TLRs. Upon ligand engagement, TLRs signal via a series of adaptor proteins, resulting in the activation of NF-κB or the interferon regulatory factors 3 and 7 (IRF-3 and IRF-7), and to the production of several pro-inflammatory cytokines, interferons, chemokines, prostaglandins and defensins[137]. Human bronchial EC lines or primary cells express large numbers of TLRs and have been shown to react functionally to various TLR ligands. However, expression levels differ among TLRs: TLRs 2–5 are highly expressed and TLRs 7–8 are expressed at low levels[138]. Apart from TLR-2 and TLR-4, no formal expression profile of TLRs by airway ECs in mice has been made, but functional studies showed that the ECs of mice react to almost all TLR agonists[139].

***NOD-like receptors.*** Members of the NOD-like receptor (NLR) family share a C-terminal leucine-rich repeat domain that interacts with PAMPs, a central nucleotide oligomerization domain (NOD) and one of three N-terminal signaling domains. Although mostly expressed by leukocytes, NOD1 and NOD2 are expressed by human airway ECs[140]. The activation of NODs results in MAPK and NF-κB–dependent production of inflammatory mediators. Some NLRs promote the assembly of the inflammasome, a molecular complex that activates caspases to convert pro–IL-1β and pro–IL-18 (and possibly pro–IL-33) into their mature secreted forms. The best known NLR in this regard is NLRP3, which was shown to induce $T_{H}2$ immune responses to crystals and the adjuvant alum[141,142]. NLRP3 is involved in responses to serum amyloid A, a mediator and biomarker of inflammation in asthma, by promoting a $T_{H}17$-type immune response[143]. The involvement of NLRP3 in pulmonary responses to environmental allergens or pollutants is not entirely clear, but in our hands, allergic airway inflammation induced by HDM, cigarette smoke or diesel did not involve the NLRP3 signaling pathway[68,144].

***C-type lectins.*** Epithelial expression of the C-type lectin receptor dectin-1 was shown to mediate recognition of β-glucan motifs in the inhaled fungus *Aspergillus fumigatus* and in several other environmental allergens such as pollens, HDMs and animal dander[32,145]. On DCs, dectin-2 seems to be the major C-type lectin receptor involved in recognition of HDM allergen[146].

***Protease-activated receptors.*** ECs secrete cytokines and chemokines in response to proteolytic allergens such as Der p 1 (refs. 147,148) and protease allergens from pollen and cockroach[149] through activation of protease-activated receptor 2 (PAR-2). Particularly when allergens are administered mucosally, PAR-2 is required for the induction of $T_{H}2$ immunity, suggesting an important role for PAR-2 in the epithelial programming of the adaptive immune response by proteolytic allergens[150].

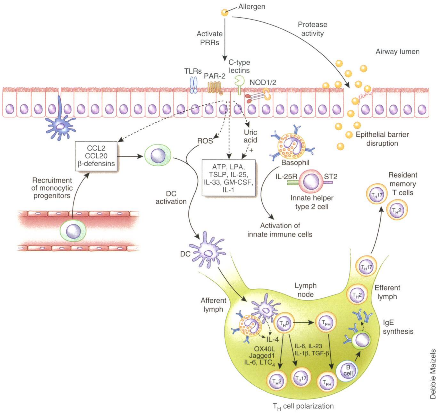
### Epithelial activation threshold influences allergic sensitization

These studies[17,18] highlighted the importance of a single TLR in generating a response to the complex allergen HDM, which contains multiple (proteolytic) allergens and microbial contaminants. Many allergens, such as Der p 2 and Der p 7 from HDM and Fel D 1 from cats, activate TLR4 signaling, which may explain why they are so potent at inducing allergic responses[19]. TLR4-mediated responses require the coordinated action of several molecules, including soluble CD14 and the TLR4 cofactor MD2. Previous studies have reported that, in the resting state, airway ECs are hyporesponsive to TLR4 agonists in comparison to myeloid cells as a result of either low expression of MD2 or intracellular localization of TLR4 (refs. 20,21). Inhaled allergens can simultaneously trigger other PRRs (**Box 1**), and it will be a challenge to untangle the intersections of the various signaling pathways involved.

The activation threshold of ECs and their resulting ability to trigger innate and adaptive responses could be promoted by prior exposure to DAMPs and PAMPs, which either increase the expression of PRRs themselves or alter the expression of signaling proteins downstream of PRRs. Viral infection of airway epithelia with respiratory syncytial virus (RSV) or exposure to cigarette smoke (which causes damage to cells and the release of reactive oxygen species (ROS)) upregulates TLR4 expression and promotes its localization to the cell membrane, thus increasing endotoxin responsiveness[22,23]. This increased sensitivity of airway ECs might explain how RSV infections early in life or exposure to cigarette smoke act as predisposing factors for allergic sensitization and inflammation.

The activation threshold of ECs can also be regulated at the level of downstream signaling cascades. Most PRR signaling pathways activate the transcription factor nuclear factor-κB (NF-κB), which controls the expression of an array of inflammatory cytokine genes[24]. The central role of NF-κB in the control of pulmonary inflammation was elucidated in mice lacking the NF-κB subunits p50 or p65, which showed reduced responses to endotoxin and allergens[25]. Later studies revealed that constitutive activation of NF-κB in airway ECs was sufficient to activate DCs, breach inhalational tolerance and promote sensitization to the harmless inhaled allergen ovalbumin (OVA)[26], whereas inhibition of epithelial NF-κB reduced $T_{H}2$ cell recruitment and airway remodeling[27].

One of the early-response genes induced by NF-κB nuclear translocation is the ubiquitin-editing enzyme A20 (also known as TNFAIP3), which deubiquitinates crucial signaling intermediates of TLR-induced activation of NF-κB and thus acts as a negative regulator of inflammatory gene induction in the epithelium and DCs[28,29]. Lung-specific overexpression of the A20 binding protein ABIN-1 suppressed allergic inflammation[30]. Additional studies are needed to address the precise mechanisms whereby NF-κB activation and deactivation are regulated in airway ECs. In particular, these control mechanisms



Debbie Maizels

**Figure 1** Influence of ECs on the induction of allergic sensitization by DCs. Inhaled allergens such as HDMs can activate PRRs on ECs and DCs and can cleave epithelial tight junctions, gaining access to the DC network. Some allergens also induce production of ROS that activate DCs or ECs through NF-κB activation. The net result of PRR activation and ROS production in ECs is the production of endogenous danger signals such as uric acid, ATP and lysophosphatidic acid (LPA), as well as the DC-activating cytokines TSLP and GM-CSF and several interleukins, including members of the IL-1 family. ECs also produce CCL2 and CCL20, which attract more DC progenitors, such as monocytes, to the lung. Under the influence of these signals, DCs migrate to the T cell area of the draining lymph nodes, where they interact with naive T cells and induce $T_H2$, $T_H17$ and T follicular helper ($T_{FH}$) differentiation. This $T_H$ polarization is heavily influenced by secreted cytokines and membrane-expressed molecules such as OX40 ligand. $T_{FH}$ cells can then promote B cell differentiation and IgE synthesis. Basophils are an important source of IL-4, which further supports $T_H2$ development initiated by DCs and promotes IgE synthesis by B cells. NOD 1/2, nucleotide oligomerization domain; PAR-2, protease-activated receptor 2.

may be susceptible to epigenetic alterations induced by environmental factors such as microbial infection and environmental pollutants. These epigenetic changes might, in turn, alter the threshold for epithelial activation in response to allergens and viruses.

## Molecular crosstalk between ECs and immune cells

There has been great progress in understanding the mechanism by which ECs influence DC function[31] (**Fig. 1**). Airway ECs produce CC chemokine ligand 2 (CCL2) and CCL20 in response to HDM inhalation, which attracts monocytes and immature DCs to the lung[17,32]. Bronchial ECs also release homodimers of the cytokine subunit IL-12p40 that are chemotactic to monocytic cells and DCs[33]. Triggering of TLR4 on ECs by HDM also induces the production of thymic stromal lymphopoietin (TSLP), granulocyte-macrophage colony–stimulating factor (GM-CSF), IL-25 and IL-33. These innate cytokines have pleiotropic effects, yet they share the propensity to activate DCs that prime $T_H2$ responses by inhibiting the production of the $T_H1$-polarizing cytokine IL-12, inducing chemokines that attract $T_H2$ cells or upregulating expression of surface molecules such as OX40L that can instruct $T_H2$ cell development.

IL-33 and other members of the IL-1 family of cytokines, such as IL-1α, IL-1β and IL-36γ (IL-1F9), are released from bronchial ECs in response to allergen exposure[34,35]. It is unclear, however, whether all of these cytokines activate DCs to promote $T_H2$ immune responses[36]. IL-33 is normally sequestered in the nucleus of ECs, but it can be secreted upon epithelial activation. IL-33 activates lung DCs and promotes their induction of $T_H2$ responses by acting on the T1 (ST2) receptor[37,38]. Blocking IL-33 during sensitization to inhaled antigens by DCs abolishes $T_H2$ development[39]. The expression of IL-33 is higher in bronchial ECs of people with asthma[40]. Although IL-25 promotes $T_H2$ immunity in the lung[41,42], its potential to activate DCs remains unclear. The proteolytic enzyme MMP7, which is released from bronchial ECs, is necessary for optimal production of IL-25 (ref. 43). A recent study demonstrated that epithelial-derived IL-25 induced Jagged 1 expression on DCs and promoted $T_H2$ responses in the lung tissue of RSV-infected mice[44].

TSLP and GM-CSF stimulate DCs to promote $T_H2$ immunity. Overexpression of these cytokines in the lungs of mice induces spontaneous $T_H2$ sensitization to the inhaled innocuous protein OVA[45,46]. Conversely, neutralization of GM-CSF abolishes sensitization to HDM and attenuates the adjuvant effects of diesel particles on allergic sensitization[47–49]. When ECs from humans who have asthma are cultured, they continually overproduce GM-CSF, suggesting that its production could be epigenetically regulated in asthma[50]. The expression of TSLP is also increased in bronchial biopsies and sputum from humans who have asthma, particularly in severe disease[51,52]. Genetic polymorphisms in the promoter region of TSLP are associated with increased risk of asthma[53]. Proteolytic allergens, diesel-exhaust particles and cigarette smoke induce epithelial production of TSLP, which causes DC activation[54,55]. The TSLP receptor (TSLPR) is expressed not only in DCs but also in human bronchial ECs. TSLP stimulates the proliferation of bronchial ECs and EC IL-13 production[52]. One caveat regarding therapeutic targeting of TSLP is that TSLP can contribute to epithelial repair by induction of secretory leukocyte protease inhibitor (SLPI) and other mechanisms[52,56]. Expression of SLPI has the potential to dampen allergic airway inflammation; therapeutic interference with TSLP might abolish this protective pathway[57].

Epithelial-derived cytokines also activate innate immune cells such as basophils, mast cells, eosinophils and innate non-T and non-B lymphoid cells (innate lymphoid cells or nuocytes)[58–61]. TSLP promotes the growth and differentiation of basophils from the bone marrow[62]. Basophils provide an early source of polarizing IL-4 to further enhance $T_H2$ development initiated by DCs[15] (**Fig. 1**), whereas nuocytes are an IL-33– and IL-25–responsive population that mainly produce IL-5 and IL-13 and could thus contribute to eosinophilia, goblet-cell metaplasia and virus-induced AHR[63,64].

Once initiated, epithelial responses to allergens and pollutants are maintained through the action of $T_H2$ effector cytokines, providing an important feedback loop that can perpetuate disease. Airway ECs respond to the $T_H2$ cytokines IL-4 and IL-13 by producing GM-CSF and the chemokines IL-8, CCL11 and CCL17, which attract neutrophils, eosinophils and CD4 $T_H2$ cells, respectively[65,66]. These effects of IL-4 and IL-13 on ECs are counteracted by the $T_H1$-type cytokine interferon-γ (IFN-γ)[67].

### Epithelial DAMPs promote airway inflammation

In addition to producing cytokines, ECs may also regulate the allergic immune response by producing DAMPs. We have previously reported that a single administration of HDM in the airways of naive mice or of people with atopic asthma induces the release of both ATP and uric acid, which activate DCs and other innate immune cells[68–70]. HDM-induced uric acid promoted $T_H2$ sensitization by amplifying EC production of TSLP, GM-CSF and IL-25. In particular, airway ECs were a source of uric acid, and the release of uric acid was TLR4 dependent. Production of uric acid is the result of an antioxidant response to ROS, which are often produced by airway ECs in response to inhalation of allergens, virus infection or environmental pollutants[71,72].

ROS comprise another type of DAMP that can activate DCs by promoting NF-κB activation[73]. The transcription factor NRF2 controls the expression of a set of antioxidant genes. Mice lacking *Nrf2* are hypersensitive to ragweed allergen and ambient particulate matter, and DCs isolated from these mice are hyperactivated[72]. The ECs of subjects with asthma produce fewer antioxidants, which could explain their increased sensitivity to allergens and environmental pollutants[11]. Exposure to allergens and pollutants can cause plasma extravasation, leading to tissue hypoxia and immediate tissue edema. In response to hypoxia, ECs upregulate the hypoxia-inducible factor-1α (HIF-1α), which leads to gene transcription and production of vascular endothelial growth factor (VEGF). Neutralization of HIF-1α or VEGF suppresses established allergic inflammation[74,75].

Other danger signals released in response to allergen exposure include lysophosphatidic acid, which activates EC release of TSLP in a process requiring the adaptor molecule CARMA3 (ref. 76) and low-molecular-weight (200-kDa) hyaluronan[77], but it is unclear at present whether expression of these DAMPs is upregulated in ECs from subjects with asthma.

### EC-derived signals can also dampen inflammation

In response to allergen exposure, ECs produce prostaglandin $E_2$ ($PGE_2$), which suppresses DC reactivity by acting on the EP4 receptor[78]. Several other lipid mediators (such as lipoxin A4, resolvins and protectins) that suppress lung inflammation are reduced in the airways of subjects with asthma[79]. After airway allergen challenge, airway ECs redistribute the heparan sulfate proteoglycan syndecan-1 from the basolateral space to the airway lumen, thus sequestering inflammatory chemokines that attract eosinophils and $T_H2$ cells[80]. The IL-1 family member cytokine IL-37 is also produced by bronchial ECs and is an inhibitor of endotoxin-mediated innate immune lung inflammation[81]. However, it remains unclear whether IL-37 expression is reduced in subjects with asthma[81]. An additional regulatory mechanism that warrants further study is gene regulation by microRNAs. Although certain microRNAs are induced by allergen challenge in mice and have the potential to dampen inflammation[82], their precise localization has not been studied. Undoubtedly, other anti-inflammatory mechanisms are employed by ECs to keep immune activation in check, and future work is essential to understand the mechanisms involved in resolution of allergic inflammation.

### Barrier function of the airway epithelium in asthma

The airway epithelium has typically been thought to function mainly as a physical barrier and in mucociliary clearance by impeding the access of allergens to lung DCs. It has therefore been proposed that the allergenicity of inhaled substances depends on their potential to disrupt epithelial barriers[83]. In the airways of individuals who have asthma, the epithelia are fragile, and some areas of epithelial basement membrane seem to be denuded of ciliated cells[84]. The integrity of the epithelial barrier depends on apical tight junctions and adherens junctions that keep bronchial ECs together and maintain their apicobasal polarity[11]. E-cadherin is a major component of adherens junctions, and its expression is reduced in biopsies from subjects with asthma, possibly as a result of epithelial-to-mesenchymal transition[85,86]. DCs receive a tonic inhibitory signal through homotypic E-cadherin interactions between DCs and ECs[87,88]. Loss of E-cadherin expression could release this inhibitory signal and facilitate allergic sensitization by activating DCs. Loss of E-cadherin in cultured ECs also enhances production of TSLP[89] and could therefore be a primary driving force in breaching inhalational tolerance to allergens in individuals with asthma.

It is difficult to address whether loss of E-cadherin is a cause or effect of allergic sensitization. However, in a linkage-analysis study in individuals with asthma, variants in the gene encoding protocadherin 1 were associated with bronchial hyperresponsiveness[90]. Protocadherins, adhesion molecules related to cadherins, are extensively regulated and expressed during differentiation of bronchial ECs, but their function has yet to be determined. The loss of E-cadherin and/or tight-junction proteins and the consequent breach in barrier function could also be due to proteolytic activity of inhaled allergens originating in HDM[83], cockroach[91], pollen[92] or fungi[93]; by exposure to environmental pollutants such as cigarette smoke[94] and ozone; or by respiratory infection with RNA viruses[95]. HDM-induced disruption of junctional proteins and barrier function may also be due to the binding of epidermal growth factor (EGF) and/or TGF-α to apically expressed EGF receptor (EGFR) on ECs[89,96] (**Fig. 2**). Recently, another molecule, septin-2, was found to regulate apicobasal polarization of ECs and to prevent disruption of lung epithelial integrity[97]. Whether septin-2 expression differs between individuals with asthma and healthy controls remains to be elucidated. It is striking that IL-4 and IL-13, some of the prominent effector molecules of $T_H2$ cells, also disrupt epithelial barrier function and might, in this way, perpetuate allergic inflammation[98].

### ECs and basement-membrane thickening

Some patients with chronic asthma show a progressive decline in lung function that is thought to be caused by structural remodeling of the airways, characterized by subepithelial fibrosis and smooth-muscle hyperplasia. Changes to the epithelial basement membrane and deposition of extracellular matrix components in the lamina reticularis lead to pseudothickening of the basement membrane, a common feature of airway remodeling (**Fig. 2**). This response is the result of communication between structural cells and immune cells. ECs release EGF, and eosinophils and myofibroblasts produce TGF-β to promote synthesis of extracellular matrix components and collagen[99,100]. This interaction between ECs and fibroblasts

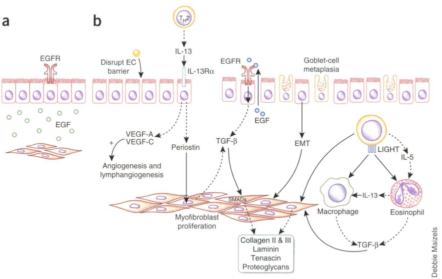


**Figure 2** Airway remodeling in the epithelial mesenchymal trophic unit. (**a**) In healthy ECs, EGF is secreted on the basolateral side, where it is sequestered from EGFR on the apical side. The sequestration is ensured by an intact epithelial barrier function. In the healthy state, the basement membrane is thin and there are few fibroblasts. (**b**) When ECs are chronically triggered by proteolytic allergens, environmental pollutants or $T_H2$ cytokines, they can produce cytokines and growth factors such as TGF-β and TGF-α. Under the influence of EC-derived VEGF-A and VEGF-C, there is neoangiogenesis and lymphangiogenesis. New lymphatics might provide a means for DCs to reach the lymph nodes, whereas newly formed vessels allow for extravasation of more inflammatory cells. Epithelial tight junctions are disrupted by proteolytic enzymes or cytokines, allowing basolateral EGF to reach the apical EGFR, inducing EC activation. ECs release periostin, which further boosts TGF-β production, activating the underlying fibroblasts to differentiate into myofibroblasts that synthesize more collagen and extracellular matrix components. Another source of TGF-β is the eosinophil, which is induced under the influence of IL-5 derived from $T_H2$ lymphocytes. Inflammatory T cell–expressed LIGHT is able to interact with eosinophils and macrophages, driving further production of cytokines. There is also some evidence for epithelial mesenchymal transition (EMT), a process whereby ECs acquire characteristics of fibroblasts and downregulate expression of E-cadherin.

is sometimes called the epithelial-mesenchymal trophic unit[101]. ECs from individuals with asthma produce high amounts of periostin, which stimulates TGF-β production and modifies collagen synthesis by airway myofibroblasts[102]. A study employing mepolizumab, an antibody that blocks IL-5, demonstrated that the ensuing reduction in eosinophils was associated with reduced basement membrane deposition of tenascin, lumican and procollagen type III (ref. 99). Studies in mice genetically deficient in eosinophils or depleted of eosinophils through the use of antibodies to Siglec-F support the idea that eosinophils control airway remodeling[103]. However, eosinophils are not always involved in remodeling. The tumor necrosis factor (TNF)-family member LIGHT is expressed on lung inflammatory cells after allergen exposure, and pharmacological inhibition of LIGHT reduces lung fibrosis, smooth-muscle hyperplasia and airway hyperresponsiveness in mouse models of chronic asthma, despite having little effect on airway eosinophilia[104]. Lack of TNFR-I (p55) or TNFR-II (p75) reduces features of airway remodeling[105]. Subepithelial fibrosis is also influenced by regulation of the coagulation cascade. The plasminogen activator inhibitor-1 (PAI-1) promotes features of airway remodeling, and an oral PAI-1 antagonist prevents airway remodeling induced by chronic allergen exposure[106].

It has been hypothesized that the thickened basement membrane constitutes a secondary barrier that compensates for the increased epithelial fragility and leakiness, thus halting entry of allergens and pathogens and their access to DCs. We have recently tested this hypothesis and found that remodeling of the epithelial basement membrane did not impair recognition of inhaled antigens by DCs; rather, it promoted sensitization to inhaled neoallergens by causing persistent activation of DCs and facilitated DC migration to the draining lymph node by promoting lymphangiogenesis[10]. In our view, the changes in the basement membrane are not the end result of chronic allergic inflammation, but could be a driving force contributing to allergic sensitization. This theory would fit with the observation that basement membrane thickening is detectable in children younger than three years old with persistent wheezing before the diagnosis of asthma[107,108].

## EC differentiation and increased mucus production

The normal airway epithelium is made up of ciliated cells, Clara cells, goblet cells and basal cells that can transdifferentiate. In individuals with asthma, some groups have reported an increase in basal ECs that express high levels of cytokeratin 5 and the cell cycle–control protein p63 but do not express E-cadherin[109]. In asthma, there is also an increase in the number of goblet cells. This increase, termed goblet-cell metaplasia (GCM), is not due to the proliferation of preexisting goblet cells but rather to the transdifferentiation of ciliated and Clara cells to goblet cells, resulting in more mucus production. Goblet cells produce the mucins MUC5B and MUC5AC, which are responsible for the viscoelasticity and hydration of the mucus covering the ciliary escalator. In asthma, the sputum is often so dry that it can lead to airway obstruction. How GCM is regulated has been the subject of numerous studies, but several molecular signaling pathways converging on a common set of transcription factors have been identified[100] (**Box 2** and **Fig. 3**).

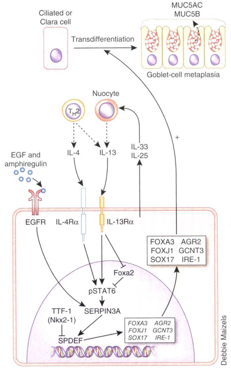
There is a link between altered epithelial barrier function and the induction of GCM. EGF is a key EC-derived cytokine that promotes GCM. EGF is normally secreted on the basolateral side of the epithelium and sequestered from its receptor on the apical side through intact adherens junctions and tight-junction barriers between ECs[110]. The EGFR is also activated by amphiregulin, which is released from damaged ECs[111]. Conversely, it seems that epithelial-barrier repair mechanisms impede GCM. Trefoil factors are upregulated with damage and are important for epithelial repair in the gut mucosa. Deficiency of trefoil factor 2 increased GCM in a mouse model of chronic asthma[112]. Increased production of mucus can, therefore, be seen as another form of compensation for a disrupted epithelial barrier.

The induction of GCM is regulated by cytokines secreted from cells of the innate and adaptive immune system (**Fig. 3**). GCM can be induced *in vitro* when air-liquid interface cultures of human bronchial ECs are exposed to the $T_H2$-type cytokines IL-4 or IL-13 or when mice receive an intratracheal injection of IL-13 (refs. 113–115).

$T_H2$ effector cells are not the only source of IL-13; innate lymphoid cells (or nuocytes) can release IL-13 in response to EC-derived IL-33 and/or IL-25 as well. Similarly to ECs, innate lymphoid cells also release amphiregulin that can bind the EGF receptor and promote airway remodeling[64,116]. Activation of the innate and adaptive arms of the immune system and induction of GCM are closely linked at the molecular level. The transcription factor Nkx2-1 suppresses the GCM master-switch transcription factor SAM pointed domain–containing Ets transcription factor (SPDEF) and suppresses the production of the proinflammatory chemokines CCL17 and CCL26 in ECs. Along the same lines, Foxa2, a forkhead box–family transcription factor involved in suppression of the GCM program, suppresses epithelial production of IL-13, IL-33 and the chemokines CCL20 and CCL17 (ref. 117). Inflammatory DCs accumulate in the lungs of *Foxa2*$^{-/-}$ mice and promote $T_H2$ immunity[15]. These findings suggest that the epithelial GCM differentiation program is closely linked to the regulation of innate and adaptive $T_H2$ immunity and that these epithelial responses co-evolved as protective mechanisms to restore homeostasis in response to (infectious) insults. Protection from parasitic infection could have been the driving pressure behind this evolution. Helminth infection of the gut induces a similar program of goblet-cell metaplasia, which is driven by SPDEF and leads to changes in the composition of the mucus barrier, recruitment of inflammatory DCs, innate lymphoid cells and eosinophils, and a $T_H2$ response leading to parasite expulsion[60,118–120] (**Box 2**).

## BOX 2 Molecular control of goblet-cell metaplasia in asthma

The genetic program controlling lung development is well characterized. An emerging concept is that this same transcriptional network can also be turned on during adult life in response to lung injury or inflammation[151,152]. This is illustrated by the molecular process controlling goblet-cell metaplasia (GCM) in asthma.



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This increase is not due to proliferation of pre-existing goblet cells but rather to the transdifferentiation of ciliated and Clara cells to goblet cells, hence the term GCM. A key discovery was that IL-13, EGF and amphiregulin induce SPDEF in a process requiring serpin 3A (the mouse homolog of human serpin B3 and serpin B4)[153]. Transgenic overexpression of *Spdef* in mouse Clara cells causes severe GCM and induces genes involved in epithelial- and goblet-cell differentiation and protein glycosylation, including members of the forkhead box family (*Foxa3* and *Foxj1*), *Sox17*, anterior gradient 2 (*Agr2*) and *N*-acetyltransferase 3 mucin type (*Gcnt3*)[154]; whereas deficiency of SPDEF abolished IL-13– and allergen-induced GCM[155]. SPDEF binds Nkx2-1 (TTF-1), a developmental transcription factor involved in lung budding from the primitive foregut. The expression of Nkx2-1 is downregulated in mucosal biopsies of patients who have asthma, whereas transgenic overexpression of Nkx2-1 in mice inhibited GCM by suppressing the expression of SPDEF[156]. Foxa2, another forkhead-box family transcription factor whose expression is restricted to bronchial ECs, also suppresses SPDEF and GCM[117].

The production of mucus is a complicated process involving multiple glycosylation reactions taking place within the endoplasmic reticulum (ER). Goblet cells in the gut activate the unfolded protein response (UPR) to deal with the increased biosynthetic demands of increased mucus production on the ER, leading to activation of the ER resident kinases inositol-requiring endonuclease-1α (IRE-1α) and IRE-1β and of the downstream transcription factor X-box–binding protein-1 (XBP-1)[121]. IRE activity is also increased in lung ECs in response to GCM induction[122], and downstream XBP-1 could influence the secretion of proinflammatory cytokines, as the XBP-1 and NF-κB pathways act synergistically to activate inflammatory gene expression[123]. The gene encoding ORMDL3 was identified as a susceptibility locus for childhood asthma[124]. ORMDL3 is a regulator of the UPR that could modify IRE-1 activity and XBP-1 induction, but it remains unclear how this influences epithelial cell biology or the GCM differentiation program[125].

### Viral exacerbations

Viral infection with rhinovirus is one of the most common triggers for asthma exacerbations, causing an increase in symptoms, mucus secretion and airway obstruction[126]. Rhinovirus normally replicates only in the ECs of the upper airways, but bronchial ECs from the lower airways of people with asthma are uniquely sensitive to viral replication. Virus can be recovered from the deeper airways during an exacerbation. This greater sensitivity could be caused by reduced production of type I IFN-β and type III IFN-λ by ECs, resulting in defective EC apoptosis upon viral infection[127,128]. More recently, studies have also focused on TLR3, a receptor for double-stranded RNA. IL-13 can inhibit production of double-stranded RNA (dsRNA)-induced IFN-λ by airway ECs, which contributes to the impaired antiviral response observed in individuals who have asthma[129]. IFN-λ not only has antiviral effects but also controls allergic inflammation directly. When administered to mice, IFN-λ2 (IL-28A) suppressed $T_H2$ cytokine release and protected mice from allergic airway inflammation, a result of its capacity to downregulate OX40L and promote IL-12 production in DCs[130]. Viral infection or dsRNA exacerbate asthma by increasing the production of IL-8 and other chemokines that attract eosinophils, $T_H2$ cells and DCs[131–133]. The epithelial barrier is also compromised after respiratory viral infection in a process that involves EGFR signaling and leads to enhanced leakiness and GCM[95].

## Conclusion and therapeutic implications

Current standard therapy for asthma consists of inhaled corticosteroids combined with a long-acting bronchodilator. However, these treatments only manage symptoms and do not alter the natural course of disease. Although inhaled steroids have shown superior anti-inflammatory effects in clinical trials with carefully selected subjects, clinical experience with these drugs in asthma has shown that many patients do not respond favorably. Patients with a $T_H2$ signature in bronchial ECs that is characteristic of IL-13 activation and comprised of the genes encoding Serpin B2, periostin and CLCA1 respond best to inhaled steroids[134]. This implies that biomarkers can be used to stratify patients before initiating therapy and increase the likelihood of a favorable clinical response using samples derived from the airways as well as from serum.

Finding new drugs that selectively inhibit crucial aspects of the epithelial-immune or the epithelial-mesenchymal interaction could potentially inhibit mechanisms driving sustained allergic inflammation. Therapeutic targeting of the cytokines involved in this network, such as TSLP, GM-CSF, IL-25, IL-33, IL-1, EGF, TGF-β and LIGHT, could reduce the risk of allergic sensitization and prevent airway remodeling. Given the importance of epithelial PRRs in promoting sensitization, therapeutic modulation of PRRs could also prove beneficial. Repeated mucosal delivery of TLR7 or TLR9 agonists has been shown to reduce airway eosinophilia and features of chronic airway remodeling in a process that resembles endotoxin tolerance[135,136]. Chronic exposure to PRR agonists could also potentially reprogram ECs to react less to environmental triggers by engaging negative regulatory mechanisms within the PRR signaling cascade or by epigenetically silencing gene expression. It is also imperative that we find ways to restore the innate epithelial response to respiratory viral infection to prevent asthma exacerbations.

Fifteen years ago, Stephen Holgate was the first to suggest, on the basis of observations in asthma biopsies and cultured bronchial ECs[101], that ECs are a major culprit in asthma. Since these initial studies, it is now clear that the airway epithelium controls many aspects of allergic sensitization and is an important player in allergic inflammation, remodeling and bronchial hyperreactivity. Thus, strategies aimed at targeting the ECs could prove beneficial in altering the natural course of disease and decreasing the burden of disease on patients.

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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nature medicine

# IgE and mast cells in allergic disease

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**Immunoglobulin E (IgE) antibodies and mast cells have been so convincingly linked to the pathophysiology of anaphylaxis and other acute allergic reactions that it can be difficult to think of them in other contexts. However, a large body of evidence now suggests that both IgE and mast cells are also key drivers of the long-term pathophysiological changes and tissue remodeling associated with chronic allergic inflammation in asthma and other settings. Such potential roles include IgE-dependent regulation of mast-cell functions, actions of IgE that are largely independent of mast cells and roles of mast cells that do not directly involve IgE. In this review, we discuss findings supporting the conclusion that IgE and mast cells can have both interdependent and independent roles in the complex immune responses that manifest clinically as asthma and other allergic disorders.**

People with allergic disorders such as atopic dermatitis (eczema), allergic rhinitis (hay fever), food allergy and allergic (or atopic) asthma can experience acute signs and symptoms of disease within minutes of exposure to the associated allergens. However, such individuals also typically develop long-term changes in the affected tissues, often called tissue remodeling, after repeated exposure to these allergens over periods of weeks to years. There is consensus that antigen-specific IgE antibodies, together with one of the major effector cells of allergy, the mast cell (**Box 1**), can be crucial for the development of the acute manifestations of these allergic disorders. But there is less agreement about the role of IgE and mast cells in the chronic, long-term tissue changes that account for much of the morbidity of these increasingly prevalent diseases.

IgE[1–3] and mast cells[4–7] have each been the topic of recent reviews. We focus here on aspects of the biology of IgE and mast cells that we think are most relevant to their proven or potential roles in allergic disorders, especially asthma. We discuss evidence indicating that IgE and mast cells, acting either individually or in concert, can have both nonredundant and partially redundant roles in the pathogenesis of chronic and acute manifestations of asthma. We also describe some approaches that are being taken to exploit our understanding of the biology of IgE and mast cells to craft better ways to manage and treat people with allergic diseases.

## Allergen sensitization and antigen-specific IgE production

The discovery and characterization of the antibody class now called IgE[8], culminating in the independent descriptions of this class of antibodies by the Ishizakas[9] and Johansson and Bennich[10], arguably represents the most crucial advance in our understanding of the immunological basis of allergic disorders. Production of antigen-specific IgE requires that such antigens are taken up by dendritic cells, B cells or other antigen-presenting cells, which, in the presence of interleukin-4 (IL-4) or IL-13 provided early in the process by one or more cell types, present the processed antigens to cognate naive T cells that then acquire a T helper type 2 ($T_H2$) cell phenotype[11] (**Fig. 1**, left). $T_H2$ cells both engage cognate B cells through B cell major histocompatibility complex (MHC) class II and co-stimulatory molecules and secrete IL-4 and IL-13, inducing B cells to undergo class-switch recombination (CSR), resulting in the variable, diverse and joining (VDJ) segments that were initially linked to another constant (C) region in the immunoglobulin heavy chain locus (for example, $C\mu$ or $C\gamma$) to instead be linked to the $C\varepsilon$ region (**Fig. 1**, right). CSR also can be induced by IL-4 and/or IL-13 that is derived from cells other than $T_H2$ cells, which may include mast cells and basophils[11–13].

Antigen sensitization was previously thought to occur primarily in lymphoid germinal centers, but IgE-producing B cells that undergo clonal selection and affinity maturation also can be generated in the respiratory mucosa[14] (**Fig. 1**, left). CSR resulting in production of IgE (in addition to IgA) also can occur in the gastrointestinal tract[15], and patients with food allergy have higher concentrations of IgE in the gastrointestinal tract than healthy individuals[15]. Such evidence supports the conclusion that IgE can be produced locally by B cells in the gut- or airway-associated lymphoid tissue, as well as in the lymph nodes, of individuals with food allergy[15], seasonal or perennial allergic rhinitis[16] or atopic or nonatopic asthma[14].

These observations suggest that much of the IgE responsible for 'organ-specific' allergic disorders may be produced locally in the affected anatomical sites, which also may be a survival niche for long-lived IgE-antibody–secreting plasma cells, and that IgE measured in the peripheral circulation may be primarily antibody that has escaped from the site of disease[15]. Indeed, concentrations of IgE in the peripheral blood are typically much lower than those of any other immunoglobulin isotype[1]. These findings also suggest that locally produced IgE may be pathogenic in at least some cases of so-called

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## BOX 1 The basics of IgE antibodies and mast cells in allergy

Antigen-dependent activation of tissue mast cells that have specific IgE bound to their surface is the central event in acute allergic reactions. IgE, the immunoglobulin isotype with by far the lowest concentration in the circulation, is unable to fix complement and has little ability to cross the placenta. Its plasma concentrations can be markedly elevated in some individuals with allergic diseases or parasite infections[1]. IgE is thought to mediate biological functions primarily by binding to FcεRI, CD23 and other receptors that are expressed on mast cells and other hematopoietic cells[1,2]. The binding of antigen-specific IgE to FcεRI sensitizes mast cells and other effector cells to release mediators in response to subsequent encounters with that specific antigen or with crossreactive antigens[1–3]. Binding of antigen-IgE immune complexes to CD23 or FcεRI can serve to amplify IgE-associated immune responses by facilitating antigen presentation through CD23 on B cells or by 'antigen focusing' through FcεRI on dendritic cells or other antigen-presenting cells, leading to the production of IgE to additional epitopes of the antigens that are contained in such immune complexes[1,2].

However, it is thought that the most crucial function of IgE in allergic diseases is its ability to sensitize mast cells to release biologically active mediators in an antigen-specific manner. Mast cells are distributed throughout virtually all vascularized tissues in vertebrates, with relatively high numbers occurring near body surfaces, including the airway epithelium[63,97]. Along with dendritic cells, mast cells are one of the first immune cells to interact with allergens and other environmentally derived substances. Unlike granulocytes, mature mast cells do not ordinarily circulate in the blood; instead, hematopoietic stem cell–derived circulating mast cell precursors migrate to the peripheral tissues, where they complete their differentiation and maturation and take up residence[79]. Mast cells are potentially long-lived cells, and their number, distribution, phenotype and function can be regulated by many factors whose local concentrations can change at the sites of innate or adaptive immune responses[78]. In response to activation by specific antigens and IgE through FcεRI or by many other endogenous or exogenous substances, mast cells can produce diverse mediators that can promote or downregulate inflammation and influence tissue remodeling and function.

nonatopic asthma (that is, asthma in which IgE-dependent allergic mechanisms are not thought to have a key role) under circumstances in which it may be difficult to detect the crucial IgE antibodies systemically and in individuals in which no triggering antigen has been identified[14].

### Amplification of the IgE response to allergens through CD23

Once an individual has developed IgE antibodies to certain antigen epitopes, multiple mechanisms can lead to a more robust and diverse IgE responses to both the original as well as other antigens. Some of these mechanisms are mediated by CD23 (ref. 1), which can be expressed on cells such as epithelial cells, B cells and myeloid cells (**Fig. 1**). CD23 is a C-type lectin that can exist in a membrane-bound form that has three lectin domain 'heads' separated from the membrane by a triple α-helical coiled-coil stalk, as well as in various soluble forms whose functions depend on whether these soluble forms are monomeric or trimeric[1,17] (**Table 1**). The CD23 sheddase, ADAM metallopeptidase domain 10 (ADAM10), is the main protease that releases soluble CD23 from the membrane-associated form[3,18]. CD23 is sometimes called a low-affinity receptor for IgE, but when the three lectin head domains of CD23 interact with a single IgE molecule, the resulting affinity constant ($K_a$) (~$10^8$–$10^9$ $M^{-1}$) approaches that of the high-affinity receptor for IgE, Fcε receptor I (FcεRI) (~$10^{10}$ $M^{-1}$)[1,17].

CD23 is thought to contribute to both positive and negative regulation of IgE production (**Table 1**), but the mechanisms responsible for this, and the role of CD23 in the pathology of allergic diseases, are not fully understood[1]. Moreover, some effects of CD23 on IgE production may be influenced by other CD23 binding partners, including CD21, which can permit CD23 to participate simultaneously in biological networks involving either the complement system or IgE, and various integrins[1]. However, certain functions of CD23 clearly have the potential to influence the biology of IgE-associated allergic disorders.

For example, CD23 on epithelial cells might amplify IgE responses by moving IgE and antigen-IgE complexes present in the lumens of the gut or respiratory tract across the intestinal[19] or respiratory[20] epithelium by transcytosis ('1' in **Fig. 1**, right), where such antigen-IgE complexes can bind to and activate FcεRI on mast cells, basophils, macrophages and dendritic cells, thereby promoting allergic inflammation, and, by inducing the secretion of IL-4 and/or IL-13 by mast cells or basophils, contribute to local IgE production ('2' in **Fig. 1**, right). IgE- and antigen-activated mast cells also release mediators such as histamine, tumor necrosis factor (TNF) and prostaglandin $D_2$ ($PGD_2$) that can, in turn, promote the maturation, functional activation and migration of dendritic cells, favoring the development of sensitization to additional antigens ('3' in **Fig. 1**, right).

IL-4 and IL-13 also can increase CD23 expression on B cells and myeloid cells[21], thereby enhancing facilitated antigen presentation (FAP). In FAP, antigen-IgE complexes bound to the CD23 that is expressed on antigen-activated B cells can favor the presentation of such antigens to cognate T cells ('4' in **Fig. 1**, right and **Table 1**). The presentation of antigen-derived epitopes initiated by the recognition of an antigen by membrane-associated B cell receptors is by definition effected only by interactions of cognate B cells with $T_H$ cells (**Fig. 1**, left). By contrast, FAP of antigens bound to secreted IgE can permit any antigen-activated, CD23-expressing B cells, regardless of the specificity of the cells' B cell receptors, to present diverse epitopes (from related or unrelated antigens) to cognate T cells (**Fig. 1**, right). Thus, FAP is an efficient mechanism for so-called epitope spreading, in which the presence of an antibody response to one epitope can ultimately result in the production of antibodies to other epitopes on the same or unrelated antigens[1].

Epitope spreading is thought to contribute to the progressive development of allergies to multiple antigens in individuals with allergy. Epitope spreading may also contribute to the 'atopic march', wherein individuals who first present in early childhood with atopic dermatitis later develop allergic rhinitis and then atopic asthma[22], and may help explain how genetic abnormalities affecting one epithelium (for example, the epidermis) can predispose to allergic disorders affecting other epithelia. Notably, a large fraction of Europeans and Asians with atopic dermatitis have loss-of-function mutations in the gene encoding filaggrin, which results in impaired barrier function of the skin[23]. Filaggrin is expressed in the epidermis of the skin and in other squamous epithelia but not in the airway or gastrointestinal mucosae[24], however, individuals with filaggrin mutations that are associated with the development of atopic dermatitis are also at increased risk for the later development of atopic asthma[25].

Additional functions of CD23 include the clearance of antigen-IgE complexes, the killing of pathogens by IgE-bearing monocytes and eosinophils[26], involvement in IgE-independent[27] and IgE-dependent[28] monocyte-mediated toxicity against target cells and, through CD23 expressed on intestinal epithelial cells, the transportation of IgE and antigen-IgE complexes directly across the intestinal epithelium[19,29], which may account for delivery of maternal IgE to the fetus by swallowed amniotic fluid[30] (**Table 1**). By moving IgE in the

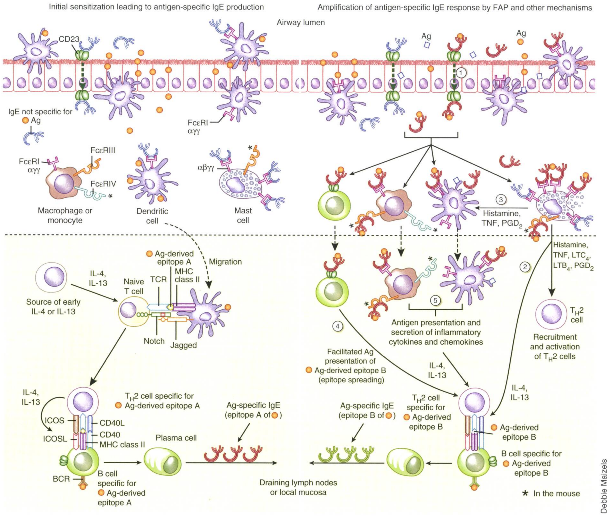
Initial sensitization leading to antigen-specific IgE production
Amplification of antigen-specific IgE response by FAP and other mechanisms
Airway lumen
CD23
Ag
Ag
1
IgE not specific for
Ag
FcεRI
αγγ
FcεRIII
FcεRI
αγγ
FcεRIV
*
Macrophage or monocyte
Dendritic cell
αβγγ
*
Mast cell
3
Histamine, TNF, PGD2
*
*
*
Histamine, TNF, LTC4, LTB4, PGD2
2
TH2 cell
Recruitment and activation of TH2 cells
Ag-derived epitope A
Migration
MHC class II
TCR
IL-4, IL-13
Naive T cell
Source of early IL-4 or IL-13
Notch
Jagged
*
*
5
Antigen presentation and secretion of inflammatory cytokines and chemokines
4
Facilitated Ag presentation of
Ag-derived epitope B
(epitope spreading)
IL-4, IL-13
IL-4, IL-13
TH2 cell specific for
Ag-derived epitope A
ICOS
ICOSL
CD40L
CD40
MHC class II
BCR
B cell specific for
Ag-derived epitope A
Plasma cell
Ag-specific IgE (epitope A of )
Ag-specific IgE (epitope B of )
Draining lymph nodes or local mucosa
TH2 cell specific for
Ag-derived epitope B
IL-4, IL-13
Ag-derived epitope B
B cell specific for
Ag-derived epitope B
★ In the mouse
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**Figure 1** Allergen sensitization and IgE production. Initial allergen sensitization results in antigen-specific IgE production (left). In individuals not yet exposed to a new environmental allergen (designated here as an antigen (Ag)), the only IgE present (blue) does not have specificity for the new antigen(s). Such IgE can be bound to the $\alpha\beta\gamma\gamma$ form of FcεRI on mast cells or to the $\alpha\gamma\gamma$ form of FcεRI on the surface of macrophages, monocytes or dendritic cells or to CD23 on airway epithelial cells or other cells (not shown here). The new antigens (orange circles) are captured by dendritic cells or macrophages in the airway lumen or in the epithelium of the airway mucosa or gain access to submucosal dendritic cells through disrupted epithelium or, for some antigens with intrinsic protease activity, by disrupting epithelial cell tight junctions. Antigen-activated dendritic cells mature and migrate to regional lymph nodes or to sites in the local mucosa, where they present processed antigen epitopes to cognate T cells; in the presence of IL-4 or IL-13, which may be derived from a variety of potential cellular sources, this induces such T cells to become differentiated and activated $T_H2$ cells. IL-4 and IL-13, which may be derived from $T_H2$ cells (shown here), basophils, mast cells and/or other sources, also activate immunoglobulin heavy chain gene CSR for antigen-specific IgE production, designated here antigen-specific IgE (epitope A), in B cells. The antigen-specific IgE response is amplified by FAP and other mechanisms (right). Antigen-specific IgE can bind to multiple cell types through various IgE receptors. Antigen-induced aggregation of IgE bound to FcεRI stimulates mast cell degranulation and the release of mediators such as histamine, $PGD_2$ and TNF, which promote recruitment of $T_H2$ cells, the migration, maturation and activation dendritic cells and antigen presentation. IgE and antigen-IgE complexes can cross the epithelium by transcytosis mediated by CD23 on airway epithelial cells (1), allowing them to bind to and activate FcεRI on mast cells and dendritic cells. This process contributes to the perpetuation of allergic inflammation and, potentially, through promotion of IL-4 and/or IL-13 secretion by mast cells (2) and effects of activated mast cells on dendritic cells (3), to additional local IgE CSR and IgE production in B cells, either to additional epitopes of the original antigen (shown here) or to new antigens bound by dendritic cells (square blue symbols). Antigen presentation mediated by binding of antigen-IgE complexes to CD23 on B cells, followed by antigen presentation by these B cells to cognate T cells (not shown here), is called FAP (4), a process that can result in epitope spreading, with production of IgE recognizing new epitopes of the original antigen (for example, epitope B, shown here) or to epitopes of new antigens, if some IgE antibodies to that antigen already exist (not shown), and the subsequent exacerbation of allergic disorders. FcεRI $\alpha\gamma\gamma$ trimers on other antigen-presenting cells (for example, dendritic cells, monocytes and macrophages) permit these cells to bind and internalize IgE that is bound to complex antigens; epitopes derived from such antigens, including those comprising epitopes for which there is not yet a specific IgE response, are then presented to cognate T cells, which, in the presence of IL-4 and/or IL-13, can become $T_H2$ cells that in turn promote the production of IgE against these new epitopes by B cells (5). ICOS, inducible T cell co-stimulator; ICOSL, ICOS ligand; BCR, B cell receptor.

**Table 1 Expression and major functions of IgE-binding receptors or molecules**

| Receptors or molecules | Cell types[a] | Major functions |
|---|---|---|
| FcεRI (αβγγ or αγγ) | **Mast cells**[38], **basophils**[38], Langerhans cells[38], **dendritic cells**[38,41], monocytes[38], **eosinophils**[38,40], **neutrophils**[39,40], platelets[138,139], bronchial epithelial cells of asthmatics[45] and airway smooth-muscle cells[44,140]. In mice: dendritic cells after Sendai virus infection[41], neutrophils and eosinophils during *Plasmodium* infection[40] and superior cervical ganglion and myenteric plexus neurons[43]; in rats: pinealocytes[141]. | αβγγ: immediate hypersensitivity[2], parasite immunity[2], enhanced cytokine production and survival in mast cells[59] and MCp recruitment in the airway[142].<br>αγγ: antigen presentation[1,38]. |
| FcεRII (CD23) | **B cells**[143], T cells, NK cells, monocytes, macrophages, **follicular dendritic cells**[143], Langerhans cells, bone marrow stromal cells, neutrophils, eosinophils, platelets[1,3,17] and airway[20] and **intestinal**[144,145] epithelial cells. | Regulation of IgE production[1], killing of intracellular pathogens (*Leishmania major*[146] and *Toxoplasma gondii*[26]) or tumor cells[28], facilitated antigen presentation[1] and transport of IgE and antigens across the epithelium[19,144]. |
| FcγRII and FcγIII (in mice[b]) | Mast cells[147] and macrophages[147]. | Cell activation[147]. |
| FcγRIV (only in mice) | Monocytes, neutrophils and macrophages[148,149]. | Phagocytosis, cytokine production and antigen presentation in macrophages[148,149]. |
| Gelactin-3 | **Mast cells**[150,151], basophils[150], neutrophils[152], **monocytes** and **macrophages**[153–156], eosinophils[157], **Langerhans cells**[158,159], T cells[160], B cells[161] and dendritic cells[162]. | Potentiate FcεRI activation[151]. |

[a]The cell types listed in bold express that receptor in both humans and mice. [b]Human FcγRIII has no affinity for IgE. However, human mast cells and basophils express the immunoreceptor tyrosine-based inhibitory motif–containing receptor, FcγRIIB, which can reduce activation of these effector cells through the FcεRI when it is co-ligated with the FcεRI[127].

opposite direction across the intestinal[31,32] or respiratory[20] epithelium, CD23 can favor the formation of antigen-IgE complexes in the lumen that can then be transported by CD23 back across the epithelium, resulting in the activation of local mast cells (or other FcεRI-bearing effector cells), a process that may both exacerbate allergic inflammation and impair mucosal epithelial function at the affected sites.

It has been reported that local IgE synthesis and expression of CD23 can occur concurrently in the fetus[30,33] and that a parental history of atopy and high titers of IgE in the cord blood are predictive of early atopy[34]. Moreover, *CD23* gene polymorphisms have been associated with effects on the development of atopy in mice and humans[35]. It is thus tempting to attribute to CD23 at least some role in the development and, through epitope spreading and other mechanisms, the exacerbation of allergic disorders.

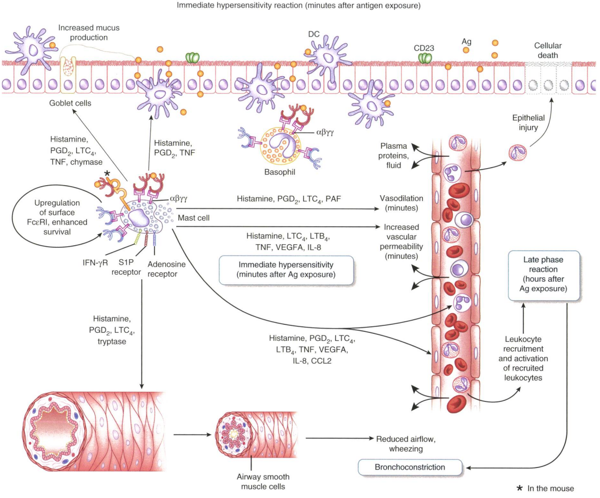
## Amplification of the IgE response to allergens through FcεRI

IgE does not fix complement and has only limited ability to cross the placenta[36], and it is thought that IgE's main biological roles reflect its ability to bind to receptors on mast cells, basophils and a variety of other cell types. The high-affinity receptor for IgE, FcεRI, as expressed by mast cells and basophils, consists of an IgE-binding α chain, in which the two extracellular domains bind IgE, a β chain, which spans the plasma membrane four times and functions as a signal amplifier[37], and two identical and largely intracellular γ chains[38]. The signaling motifs of this αβγγ form of FcεRI consist of immunoreceptor tyrosine-based activation motifs, one in the β chain and one in each of the γ chains[38]. An αγγ form of the receptor can be expressed on a variety of other cell types, including macrophages, dendritic cells, eosinophils, platelets and neutrophils[38–40] (**Table 1** and **Fig. 1**).

Although it was once thought that such widespread expression of the αγγ form of FcεRI among hematopoietic cells was more characteristic of humans than of mice, it is now known that mouse dendritic cells[41,42], eosinophils[40] and neutrophils[40] can upregulate expression of this receptor during certain immune responses. Moreover, expression of the αβγγ form of FcεRI has been detected in mouse superior cervical ganglion and myenteric plexus neurons[43] and in human airway smooth muscle cells[44]; FcεRI has also been reported on bronchial epithelial cells of asthmatic but not healthy individuals[45] (**Table 1**). Although the *in vivo* importance of such non-hematopoietic expression of FcεRI has not yet been determined, these observations suggest that IgE and FcεRI may be able to induce functional changes in such structural cells directly rather than only indirectly through IgE-driven functions of FcεRI-bearing myeloid cells.

Interactions of IgE with FcεRI-bearing myeloid cells can have multiple effects with the potential to augment production of antigen-specific IgE. Antigens can be captured (or 'focused') by IgE bound to the αγγ form of FcεRI on the surface of cutaneous Langerhans cells and other dendritic cells, which then can migrate to regional lymph nodes or perhaps to local sites in the submucosa where they can present the processed antigens to cognate naive T cells, generating $\mathrm{T_H2}$ cells[46] ('5' in **Fig. 1**, right). Although such Langerhans and dendritic cell functions can be expressed in the absence of mast cells, data from mast-cell–deficient mice[47–50] and mast-cell–knockin mice (genetically mast-cell–deficient mice that have been engrafted with mast cells)[47,48] indicate that mast cells have the potential to enhance these processes by promoting Langerhans and dendritic cell migration and, perhaps, by effects on the maturation and function of these two types of cells ('3' in **Fig. 1**, right).

In addition to immunologically 'arming' mast cells and basophils to undergo antigen- and IgE-mediated activation for mediator release, IgE binding to FcεRI also stabilizes the receptor's expression on the surface of the cells[51], thereby increasing the numbers of FcεRI on the cell surface[52–54]. This may largely account for observations showing that humans and mice with high circulating concentrations of IgE have large numbers of FcεRI on the surface of their basophils[55,56] and mast cells[54,57]. IgE-dependent upregulation of mast-cell FcεRI surface expression allows the cells to bind more IgE, which can enable the cells to respond to a larger number of different antigens, to release mediators at lower concentrations of antigens and, perhaps, to secrete certain mediators that may not be detectably released by cells with lower numbers of surface FcεRI[54,58,59]. For example, when the cells were incubated for 4 d with IgE at 5 $\mu$g ml$^{-1}$, the numbers of FcεRI on mouse mast cells derived from bone marrow[54] or human mast cells derived from umbilical cord blood[58] increased approximately 30-fold or doubled, respectively. In the human mast cells, the increase in surface expression of FcεRI was associated with increases of 73% and 156% in the amount of histamine and leukotriene C4 ($\mathrm{LTC_4}$), respectively, secreted by the cells after challenge with antibodies to IgE[58]. Thus, IgE-dependent upregulation of mast-cell FcεRI surface


Immediate hypersensitivity reaction (minutes after antigen exposure)
Increased mucus production
DC
CD23
Ag
Cellular death
Goblet cells
Epithelial injury
$\alpha\beta\gamma\gamma$
Basophil
Histamine, PGD$_2$, LTC$_4$, TNF, chymase
Histamine, PGD$_2$, TNF
Plasma proteins, fluid
*
Upregulation of surface FcεRI, enhanced survival
$\alpha\beta\gamma\gamma$
Mast cell
Histamine, PGD$_2$, LTC$_4$, PAF
Vasodilation (minutes)
Histamine, LTC$_4$, LTB$_4$, TNF, VEGFA, IL-8
Increased vascular permeability (minutes)
IFN-γR
S1P receptor
Adenosine receptor
Immediate hypersensitivity (minutes after Ag exposure)
Late phase reaction (hours after Ag exposure)
Histamine, PGD$_2$, LTC$_4$, tryptase
Histamine, PGD$_2$, LTC$_4$, LTB$_4$, TNF, VEGFA, IL-8, CCL2
Leukocyte recruitment and activation of recruited leukocytes
Reduced airflow, wheezing
Bronchoconstriction
Airway smooth muscle cells
* In the mouse

Debbie Maizels

**Figure 2** The early, immediate hypersensitivity, phase of antigen-induced airway inflammation. The individual IgE molecules that are bound to FcεRI molecules on a single mast cell can be specific for different antigens (red and blue IgE symbols). Binding of IgE to FcεRI $\alpha\beta\gamma\gamma$ on mast cells, which are normally located in airway tissues, and basophils, if they have been recruited from the blood to airway tissues, upregulates FcεRI surface expression and sensitizes these cells to respond when later exposed to specific antigens, and, in mast cells, some IgE molecules can enhance cytokine production and survival. The recognition of a particular bivalent or multivalent antigen by at least two IgE molecules bound to adjacent FcεRI molecules induces FcεRI aggregation, activating mast cells (and basophils, if they are present in airway tissues) to initiate an immediate hypersensitivity response by secreting preformed mediators and lipid mediators within minutes of antigen exposure. These mast cells also upregulate the production of many cytokines, chemokines and growth factors. Within minutes of exposure, the rapidly secreted mediators lead to bronchoconstriction, vasodilation, increased vascular permeability and increased mucus production. Mast cell mediators produced rapidly after antigen challenge can also promote dendritic cell migration, maturation and function and can contribute to the transition to the late phase reaction by promoting an influx of circulating leukocytes, both by upregulating adhesion molecules on vascular endothelial cells (for example, through TNF) and by secreting chemotactic mediators (such as LTB$_4$ and PGD$_2$) and chemokines (such as IL-8 and CC-chemokine ligand 2 (CCL2)). These recruited leukocytes can induce further inflammation and bronchoconstriction during the late phase reaction. PAF, platelet activating factor; IFN-γR, IFN-γ receptor; VEGFA, vascular endothelial growth factor A.

expression is a potentially important positive amplification loop of allergic disease in individuals who develop increased concentrations of circulating or tissue IgE.

## IgE and mast cells mediate an immediate hypersensitivity response

In an allergic person, whose tissue mast cells and other cell types already have antigen-specific IgE bound to FcεRI, re-exposure to the original or a crossreactive bivalent or multivalent antigen results in the crosslinking of adjacent FcεRI-bound IgE and the consequent aggregation of surface FcεRI. When the FcεRI aggregation is of sufficient strength and duration, it triggers mast cells and basophils to initiate complex signaling events that ultimately result in the secretion of a diverse group of biologically active products[2,4,6,38,60–63]. Some products, such as those stored preformed in the cells' cytoplasmic granules, for example, histamine, serotonin (in rodents to a much greater extent than in humans), proteases such as tryptase, chymase and/or carboxypeptidase A3 and proteoglycans (heparin and/or chondroitin sulfates), as well as newly formed lipid-derived mediators, for example, PGD$_2$, LTB$_4$, LTC$_4$, LTD$_4$ and LTE$_4$ and certain cytokines, are released by mast cells within minutes of antigen exposure. Others, including a diverse spectrum of cytokines, chemokines and growth factors, are produced in mast cells from new transcripts and are therefore secreted over a period of hours after the initial mast cell activation. Studies in mice suggest that, in addition to IgE, immunoglobulin light chains can also mediate antigen-specific mast cell activation, although the receptor responsible for this effect has been elusive[64].

## BOX 2 Late phase reactions and chronic antigen exposure lead to persistent allergic inflammation and tissue remodeling

Mast cells previously activated during the early phase reaction secrete mediators that can orchestrate the recruitment, tissue infiltration and functional activation of circulating leukocytes, including granulocytes such as eosinophils, basophils and neutrophils, as well as monocytes and T cells[66,80,163] (**Fig. 2**), which substantially increases the diversity of the cellular drivers of inflammation at the site of antigen challenge. Mast cells may therefore be a crucial source of mediators contributing to the initiation of late phase reactions[164,165]. Other sources include antigen-specific T cells, as well as myeloid cells activated by antigen-containing immune complexes if these cells are present at the site of antigen challenge, such as in individuals with ongoing airway inflammation[163]. Indeed, low levels of ongoing inflammation in the airways, as can be observed in individuals with asthma even during treatment, together with exposure to antigen peptides that are recognized by effector T cells—but not IgE antibodies—may account for the development of late phase responses in individuals without previous detectable early phase reactions[163,166].

As people afflicted with allergic asthma are usually exposed repeatedly to the antigens that elicit their allergic reactions, and this may occur over periods of years, their airways have experienced many antigen-induced early and late phase reactions. However, in addition to developing inflammation, the airways of individuals with asthma also show structural changes, called airway remodeling, which include increased numbers of mucus-producing goblet cells in the epithelium, evidence of repair responses at sites of epithelial injury, thickening of the smooth muscle layer and changes in connective tissues, blood and lymphatic vessels, mucus glands and nerves[66,167]. Persistent airway inflammation, a key feature of both allergic and non-allergic forms of asthma, probably contributes in a crucial way to tissue remodeling in asthma[167,168]. Evidence suggests that IgE and mast cells can substantially contribute to chronic airway inflammation and tissue remodeling in asthma by functioning both in a single pathway—interdependently through antigen- and IgE-dependent mast cell activation—and independently (**Fig. 3**).

In aggregate, mediators released shortly after antigen- and IgE-induced mast cell degranulation induce a response termed an immediate hypersensitivity (or 'early phase') reaction within minutes of their release. If localized to the airways, this response is characterized by increased vascular permeability, contraction of the airway smooth muscle and enhanced secretion of mucus (**Fig. 2**), resulting in acutely reduced airflow and wheezing. If the response is systemic, it can result in anaphylaxis, a catastrophic immune response that can rapidly result in death if not properly treated[65]. The inflammation and functional changes associated with early phase responses to antigens typically resolve within a few hours. However, in some individuals, a second phase of inflammation, called the late phase reaction, develops at the site of antigen challenge, typically beginning a few hours after antigen exposure.

### The IgE–mast-cell axis in chronic allergic inflammation

Mast cells activated by IgE and specific antigens produce mediators that drive early phase reactions (**Fig. 2**) and contribute to late phase reactions (**Box 2**), but these mast cells also secrete diverse cytokines, chemokines and growth factors that have the potential to influence airway remodeling[4–7,63,66] (**Fig. 3**). Compared to wild-type mice, mice lacking the Fc$\varepsilon$RI $\alpha$ chain showed diminished airway inflammation, as reflected in decreased numbers of eosinophils in the bronchoalveolar lavage fluid, in an asthma model[67]. Studies in wild-type, genetically mast-cell–deficient and mast-cell–knockin mice indicated that activation of mast cells through the FcR $\gamma$ chain (which in mice is required for mast cell activation by antigen-IgG1 immune complexes through Fc$\gamma$RIII, as well as for signaling through Fc$\varepsilon$RI) is required for the full development of many features of allergic inflammation and tissue remodeling in a model of chronic asthma[68,69]. However, many other effector cells also have the potential to contribute to these features of asthma, including antigen-specific effector T cells[70], and the relative roles of mast cells compared to other effector cells in some of these settings is not fully resolved, particularly in human subjects.

In addition to possible redundancy in the roles of mast cells and other cell types in the chronic changes associated with asthma, there is evidence that many factors can modify mast cell function in this setting. For example, *in vitro*[58,71–76] and *in vivo*[69,76,77] evidence indicates that the extent of antigen- and IgE-dependent mast cell activation may be influenced substantially (or 'tuned') by microenvironmental factors that affect the expression or function of surface receptors or signaling molecules that contribute to the positive or negative regulation of such responses[4,6,7,78,79]. Tuning factors that can be present locally at the sites of allergic inflammation, such as in the airways and other anatomical sites, include adenosine[75], sphingosine-1 phosphate (S1P)[76], certain chemokines[77] and a variety of cytokines, such as IL-4 (refs. 58,71), IL-33 (refs. 72–74) and interferon $\gamma$ (IFN-$\gamma$)[69]. Tuning also can be accomplished by cell-cell interactions. For example, interactions of mast cells and T cells can be bidirectional and complex[79,80] and include the ability of IgE-activated mast cells to enhance proliferation and cytokine production in multiple T cell subsets[81,82] and the ability of CD4$^+$CD25$^+$ regulatory T ($T_{reg}$) cells to suppress IgE-dependent mast cell activation through interactions between tumor necrosis factor receptor superfamily, member 4 (OX40), either as expressed by $T_{reg}$ cells[83] or in a soluble form[84], and the OX40 ligand (OX40L) expressed on mast cells (**Fig. 3**).

In addition to stabilizing expression of Fc$\varepsilon$RI on the mast cell surface and sensitizing mast cells to respond to specific antigens, IgE antibodies can have effects on mast cell survival or function[59,85] that seem to be independent of the presence of the antigen for which the IgE has specificity. Such findings suggest that certain IgE antibodies might favor the expansion of mast cell numbers or have effects on mast cell function *in vivo*, including increasing their secretion of cytokines and chemokines, even in the absence of a specific antigen. In addition to binding to Fc$\varepsilon$RI or CD23, IgE, and Fc$\varepsilon$RI itself, can be bound by $\beta$-galactose–containing olilgosaccharide chains of galectin-3, permitting galectin-3 to activate mast cells and basophils through carbohydrate interactions that crosslink receptor-bound IgE, Fc$\varepsilon$RI or both[86] (**Table 1**). Studies in mice lacking galectin-3 support the notion that galectin-3 can amplify the pathology that is observed in models of asthma[87] or atopic dermatitis[88].

### Independent roles of IgE and mast cells in allergic inflammation

By contrast, some potentially key effects of IgE or mast cells in allergic disease seem not to require direct interactions between these two effector elements. For example, IgE-dependent antigen focusing on dendritic cells and IgE-dependent FAP are thought to occur independently of mast cells (**Fig. 1**, right). Antigen- and IgE-dependent activation of other effector or immunoregulatory cells, including basophils, that can produce a spectrum of mediators partially overlapping with those of mast cells[89,90], does not require mast cells, except perhaps for helping to recruit such cells to sites of disease, and mast cells are not required for the actions of IgE that are mediated through CD23 on other cell types.

Mast-cell–mediator secretion can be directly activated by diverse stimuli independently of IgE and specific antigens, and many of these factors are known to be present locally at sites of allergic inflammation (**Fig. 3**). Different mast cell populations show different patterns of expression of receptors for pathogen-associated molecular patterns, including Toll-like receptors (TLRs)[4–7], and activation of mast cells through different TLRs can induce these cells to secrete distinct patterns of cytokines or chemokines[4–7]. Many additional stimuli can directly activate mast cells and, in some cases, also enhance IgE-dependent mast cell activation, including adenosine[75], S1P[76], thymic stromal lymphopoietin (TSLP)[91], IL-33 (refs. 72–74) and many other cytokines, as well as proteases,

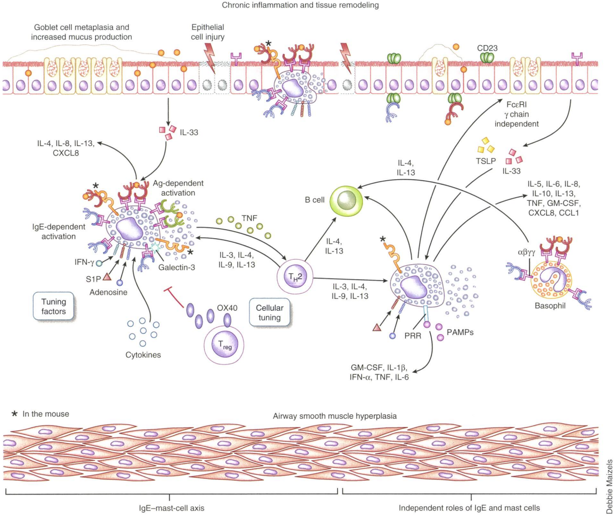

Chronic inflammation and tissue remodeling
Goblet cell metaplasia and increased mucus production
Epithelial cell injury
CD23
FcεRI γ chain independent
IL-33
IL-4, IL-8, IL-13, CXCL8
TSLP
IL-33
IL-4, IL-13
IL-5, IL-6, IL-8, IL-10, IL-13, TNF, GM-CSF, CXCL8, CCL1
Ag-dependent activation
B cell
IgE-dependent activation
TNF
IL-4, IL-13
αβγγ
IFN-γ
S1P
Adenosine
Galectin-3
IL-3, IL-4, IL-9, IL-13
T_H2
IL-3, IL-4, IL-9, IL-13
Basophil
Tuning factors
OX40
Cellular tuning
T_reg
PRR
PAMPs
Cytokines
GM-CSF, IL-1β, IFN-α, TNF, IL-6
★ In the mouse
Airway smooth muscle hyperplasia
IgE–mast-cell axis
Independent roles of IgE and mast cells
Debbie Maizels

**Figure 3** Roles of IgE and mast cells in chronic airway inflammation and tissue remodeling. In chronic allergic inflammation, repetitive or persistent exposure to allergens can result in both the production of IgE against multiple antigen epitopes of several different antigens (**Fig. 1**, right) and the development of long-term changes in the involved tissues (**Box 2**), including changes in mast cell number, tissue distribution (with mast cells in the epithelium and the smooth muscle layer, not shown here) and phenotype. Moreover, repetitive epithelial injury caused by chronic allergic inflammation can be exacerbated by exposure to environmental factors or pathogens such as viruses or bacteria, and the consequent repair response results in epithelial and mesenchymal changes that are thought to sustain $T_H2$ cell–associated inflammation, promote sensitization to additional allergens or allergen epitopes (for example, epithelial-cell–derived TSLP can upregulate the expression of co-stimulatory molecules such as OX40, CD40 and CD80 by dendritic cells, not shown here) and regulate the airway remodeling process. These processes in turn result in many functionally relevant changes in the structure of the affected tissue. There is evidence that many of these changes can be influenced by IgE and mast cells, either acting in concert through the IgE–mast-cell axis or independently. For example, both soluble factors, such as IFN-γ, S1P, adenosine and IL-33, and cells present at the site, such as $T_H2$ cells and $T_{reg}$ cells (which can interact with OX40L on mast cells) can modulate, or tune, IgE-dependent mast cell activation, and some pathogen-associated molecular patterns (PAMPs) and cytokines, including TSLP and IL-33, can activate mast cells independently of IgE to produce different spectra of cytokines or chemokines. Studies in mast-cell–knockin mice have indicated that some actions of mast cells, such as increasing the number of epithelial goblet cells, can occur in a model of chronic asthma by mast-cell–dependent mechanisms that do not require mast cell signaling through the FcεRI γ chain, whereas mast cells must express both the FcεRI γ chain and the IFN γ receptor 1 (IFN-γR1) to mediate increases in lung eosinophils, neutrophils and collagen (not shown here). Amplification of the IgE response by IgE, for example, by FAP (**Fig. 1**, right) and IgE- and antigen-dependent activation of basophils after their recruitment to the airways can occur independently of mast cells. PRR, pattern recognition receptor; GM-CSF, granulocyte-macrophage colony-stimulating factor.

inflammatory mediators, products of complement activation and exogenous agents, including bacterial toxins[4–7]. Thus, diverse mechanisms, not just stimulation with IgE and specific antigens, can elicit mast cell activation and mediator release in individuals with asthma and other allergic disorders, and the interactions among these various activation pathways in the setting of allergic disease may be quite complex.

For example, evidence from mast-cell–knockin mice suggests that the marked increase in the number of airway goblet cells observed in a model of chronic asthma can occur by mechanisms that are mast-cell–dependent but that do not require that the mast cells express the FcεRI γ chain[68,69] (**Fig. 3**). Although the relevance of these findings to human asthma has not been determined, gene set enrichment analyses have indicated that the changes in gene expression in the lung that are associated with this model of chronic asthma in mice[69], in which many of the key features seem to require mast cells for full expression[68,69], are similar to the mRNA changes observed in bronchial biopsy specimens obtained from a small group of patients with mild asthma[92].

In a different model of asthma, evidence obtained using mice lacking the mast-cell–associated chymase, mast cell protease 4 (MCPT4), indicates that the local secretion of this protease by mast cells, presumably in response to their activation by IgE and specific antigens and/or other mechanisms, can diminish the airway hyperreactivity, airway inflammation and airway smooth muscle thickening that is associated with this model[93]. These provocative findings suggest that at least some mast-cell–derived mediators may have functions that can curtail the extent of pathology at the sites of chronic allergic disease.

However, defining the roles of mast cells in asthma and other disorders could well be characterized as a moving target. For example, analyses of airway epithelial brushings or endobronchial biopsies[94–96], or lungs obtained at autopsy[97,98], from different groups of patients have documented changes in the number, distribution and/or phenotype of mast cells in asthmatic airways (**Fig. 3**). Such changes include increased numbers of mast cells in the airway smooth muscle of some individuals with clinically mild asthma[63,94], a significant trend to higher numbers of chymase-positive mast cells in the proximal airway epithelium of individuals with more severe asthma (such chymase-positive intraepithelial mast cells were rarely seen in healthy individuals)[96] and a significant trend toward lower levels of submucosal mast cells in the proximal airways, but a significantly higher ratio of chymase-positive mast cells to total mast cells, with increasing severity of asthma[96]. In a group of individuals with mild to moderate asthma, those with high expression of IL-13–responsive genes in their epithelial brushings ('$T_H$2-high asthmatics') had significantly higher numbers of intraepithelial mast cells than healthy controls or those individuals in the '$T_H$2-low' group, and these mast cells were positive by immunohistochemistry for tryptase and carboxypeptidase A3 (CPA3) but not chymase[95]. Morphological studies of autopsy specimens revealed evidence for higher levels of degranulation of mast cells in the airway smooth muscle of individuals with fatal asthma than in individuals without asthma or those with non-fatal asthma[97], as well as increased numbers of neutrophils and degranulated mast cells in submucosal glands[98]. By altering the proximity of airway mast cell populations to key airway structural cells that are targets of mast-cell–derived mediators, and possibly by changing the reactivity of mast cells to various stimuli of mast cell activation, such perturbations of airway mast cell populations may influence the extent to which mast cells contribute to airway functional and structural changes in asthma[63,94–98] (**Fig. 3**).

Why do such changes in mast cell populations occur in asthma? Many growth factors, cytokines and chemokines, some of which can be derived from mast cells, as well as other sources, can positively or negatively influence the number, phenotype and tissue distribution of mast cells, including the main survival and developmental factor for human and mouse mast cells, the c-KIT ligand stem-cell factor; many of these factors have been identified at sites of allergic inflammation in asthma and other allergic disorders[4,6,7,62,63,79,99]. However, which of these factors are most crucial in altering mast cell populations in the airways of patients with asthma *in vivo* has not yet been determined.

## Therapeutic approaches targeting IgE or mast cells

Therapeutic approaches in allergic diseases have for many years included attempts to target particular mediators that can be derived from mast cells and, in many cases, also from other cells types[100]. In addition, corticosteroids, whose effects can suppress many proinflammatory pathways[101], including some that may depend on mast cell functions such as cytokine production[102–104], can ameliorate disease in many individuals with asthma and other allergic disorders[101]. However, although such approaches have been useful in many patients, there are major unmet therapeutic needs, particularly in asthma[100].

Accordingly, given their role as key drivers of the pathology in allergic disorders, efforts are underway to target IgE and mast cells. Years of clinical experience with omalizumab, a humanized monoclonal antibody to IgE, have shown that it can provide benefit in some patients with moderate to severe asthma[105,106], as well as in some individuals with intermittent (seasonal) and persistent (perennial) allergic rhinitis[105], food allergy[107] or atopic dermatitis[108]. Omalizumab also has been reported to provide clinical benefit in a small group of patients with chronic autoimmune urticaria (a disorder characterized by recurrent episodes of hives that are resistant to antihistamine treatment and in which many patients have autoantibodies to the FcεRI α chain or to IgE itself)[109], in one patient with a severe case of apparently idiopathic cold-induced urticaria[110] and in a multicenter, randomized, double-blind, placebo-controlled study of patients with chronic urticaria who had IgE autoantibodies against thyroperoxidase[111]. These findings strongly implicate IgE and/or activation of the mast cell FcεRI in these disorders.

Much of the benefit of omalizumab treatment is thought to reflect its ability to reduce the concentration of free IgE in the blood, which can in turn lower the expression of FcεRI on mast cells and basophils and perhaps other cell types[56,57,112]. Other effects, such as downregulation of IgE-committed B cells, may also contribute to the efficacy of the drug[113]. However, not all patients with asthma are helped by such treatment, whether because of difficulty in lowering IgE concentrations sufficiently at the sites of disease or for other reasons[114], and such treatment is expensive; both of these factors limit the utility of omalizumab. Nevertheless, clinical experience with this agent supports the conclusion that IgE can have a key role in the pathology that is associated with asthma in some patients, fueling efforts to devise more effective and less costly ways to target IgE in allergic disease, including attempting to reduce or eliminate IgE using a synthetic IgE peptide vaccine[115,116].

Efforts also are underway to interfere with the binding of IgE to FcεRI. Such work includes the design of small peptides that can block this binding[117–120]. Other blocking peptides have been identified using a combinatorial chemistry approach[121], phage display[122–124] or modifications of known IgE receptor agonists[125,126]. Some of these

IgE-blocking peptides can inhibit mast cell activation *in vitro*[120,121,126] and IgE-dependent passive cutaneous anaphylaxis reactions *in vivo*[121]. In addition, there is preclinical evidence that it may be possible to achieve therapeutic benefit in IgE-associated disorders by enhancing the negative regulation of signaling through the FcεRI, for example, through the use of fusion proteins that co-ligate the FcεRI with the immunoreceptor tyrosine-based inhibitory motif–containing receptor, FcγRIIB, that is expressed on both mast cells and basophils[127].

Agents that target tyrosine kinases associated with mast cells, as well as with other cell types, are also being investigated, including those with activity against c-KIT[128–131] or spleen tyrosine kinase (SYK)[132,133]. Among the questions that need to be answered in future work targeting mast cells with these or other, perhaps more specific, agents are: can mast cells be targeted with high specificity and/or perhaps locally at sites of disease, can mast cells be eliminated without activating the cells to release clinically relevant amounts of mediators[134], and is there sufficient redundancy of the effector mechanisms in the beneficial innate and adaptive immune responses to which mast cells contribute such that mast cell numbers or functions can be safely ablated or reduced? As with any new therapeutic approach, careful studies will be required to assess whether the benefits of mast-cell–targeted treatments in selected patients outweigh any potential risks of adverse effects.

## Conclusions

Despite many years of intense study, the roles of IgE and mast cells in asthma and other allergic diseases are not fully understood. This is particularly true regarding possible beneficial roles of certain mast cell products in these disorders. However, there is strong evidence that antigen-, IgE- and FcεRI-dependent activation of mast-cell–mediator secretion can influence the pathology of allergic disorders and that the consequences of this process can either directly affect structural cells residing in the affected tissues or influence the pathology indirectly through effects of mast cells on dendritic cells, T cells, B cells and other hematopoietic cell types. Other potentially key roles of IgE and mast cells in allergic disorders may reflect the actions of each of these two effectors that can be performed largely independently of the other, such as IgE-facilitated antigen presentation and epitope spreading and the effects of mast cells on certain aspects of tissue remodeling. However, IgE and mast cells are not the only contributors to pathology in allergic disease, and both clinical studies and analyses of animal models have indicated that there can be considerable redundancy in the innate and adaptive immune mechanisms that are at work in this setting.

We suggest that an appreciation of the complexity of allergic disorders, including the potential redundancy of the effector mechanisms that drive their pathology, has clear implications for efforts to prevent or treat such diseases. To the extent that it is possible to identify factors that can favor or suppress the development of allergic disorders, efforts should be made to avoid or reduce the effects of the former and exploit the latter to help genetically susceptible persons avoid becoming sensitized to allergens in the first place. For those individuals in whom these disorders have already developed, it would be useful to have reliable, objective criteria that have been independently confirmed in large cohorts of subjects and that can be used to subcategorize patients according to the most dominant pathways driving their disease, with particular attention being paid to those that are amenable to therapeutic manipulation. Such efforts to identify subsets of patients who may respond well to individualized pathway-targeted treatment are already underway for asthma[135–137].

It seems to us that although efforts to define therapeutically relevant subsets of patients with allergic diseases are important and should continue to be pursued, it is probable that such work will confirm that IgE and mast cells contribute substantially to disease development, progression and organ-localized pathology in many people afflicted with asthma and other allergic disorders. In such individuals, we propose that targeting only IgE or mast cells may not produce as much benefit as effectively and judiciously targeting both. Such combination approaches would have the advantage of encompassing the pathological roles of IgE that do not involve mast cells and vice versa, as well as targeting the antigen-IgE-FcεRI–mast-cell axis that long has been considered a major driver of the pathology of allergic disease.

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# T cell homing to epithelial barriers in allergic disease

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**Allergic inflammation develops in tissues that have large epithelial surface areas that are exposed to the environment, such as the lung, skin and gut. In the steady state, antigen-experienced memory T cells patrol these peripheral tissues to facilitate swift immune responses against invading pathogens. In at least two allergy-prone organs, the skin and the gut, memory T cells are programmed during the initial antigen priming to express trafficking receptors that enable them to preferentially home to these organs. In this review we propose that tissue-specific memory and inflammation-specific T cell trafficking facilitates the development of allergic disease in these organs. We thus review recent advances in our understanding of tissue-specific T cell trafficking and how regulation of T cell trafficking by the chemokine system contributes to allergic inflammation in mouse models and in human allergic diseases of the skin, lung and gut. Inflammation- and tissue-specific T lymphocyte trafficking pathways are currently being targeted as new treatments for non-allergic inflammatory diseases and may yield effective new therapeutics for allergic diseases.**

## Sensitization to allergens and epithelial-barrier dysfunction

Atopic sensitization commonly occurs early in life if epithelial barrier integrity is compromised and the epithelium becomes aberrantly activated, which may occur through a complex interplay of environmental insults and host factors[1]. For example, filaggrin loss-of-function mutations that compromise epithelial barrier integrity have been associated with an increased risk for developing eczema and asthma[1,2]. Barrier dysfunction in the lung and skin allows allergens to activate the epithelium and produce cytokines that are permissive for the induction and development of T helper type 2 ($T_H2$) responses, such as interleukin-33 (IL-33), thymic stromal lymphopoietin (TSLP) and IL-25 (**Fig. 1**)[1–10]. A dysfunctional epithelial barrier also allows antigen-sampling dendritic cells to become directly activated by allergens and undergo maturation in a $T_H2$-permissive milieu such that they subsequently prime allergen-specific $T_H2$ responses[1,6,8].

## Programmed T cell trafficking and allergic disease

Accurate and efficient tissue-specific trafficking between the circulation, lymphoid organs and peripheral tissues is a fundamental prerequisite for effector T cell function and results in either host immunity or tissue immunopathology. Leukocytes coordinately use adhesion molecules in a highly regulated process to directionally extravasate from the blood into target peripheral tissues[11]. These adhesion molecules include selectins, integrins and chemoattractant receptors. Selectins are a conserved family of C-type lectins and include L-selectin and P-selectin glycoprotein ligand 1 (PSGL1) and promote the rolling movement of leukocytes along the surfaces of endothelial cells[11]. Integrins are a family of two-chain type I transmembrane receptors and include leukocyte function-associated antigen 1 LFA-1 (CD11a-CD18 or $\alpha_L\beta_2$) and $\alpha_4\beta_7$ and are involved in the firm adhesion of leukocytes to endothelial cells[11]. Chemoattractant receptors are a family of G protein–coupled receptors (GPCRs) that activate integrins through inside-out signaling to initiate firm adhesion and induce directed cell migration through tissue gradients of their ligands and include chemokine and lipid chemoattractant receptors[11]. During inflammation, ligands for selectins, integrins and chemoattractant receptors are upregulated in tissue and vascular beds and provide directional cues for inflammatory T cells, on which the corresponding receptors are upregulated, to enter inflamed tissue from the blood. In addition to inflammation-regulated trafficking cues, allergy-prone organs such as the skin and the small intestine provide an additional level of specificity in T cell trafficking to these organs through a process known as 'imprinting'[11–13].

The profile of integrins, selectins and chemokine receptors expressed by T cells is determined by their states of activation, polarization and differentiation, as well as their initial tissue site of antigen priming, leading to tissue-specific imprinting in some organs[13–16]. Tissue-specific imprinting is best characterized in the small intestine and the skin, as preferential homing to these organs was discerned more than two decades ago[17–21]. Naive, effector and memory T cells have distinct trafficking patterns within the circulation, lymphoid organs and peripheral tissue (**Fig. 1**). After T cell receptor activation, naive T cells differentiate into effector T cells (**Fig. 1**), which express an altered profile of trafficking receptors; some of these trafficking receptors are maintained in steady-state memory T cells after inflammation is resolved[14,15]. During the initial priming of naive

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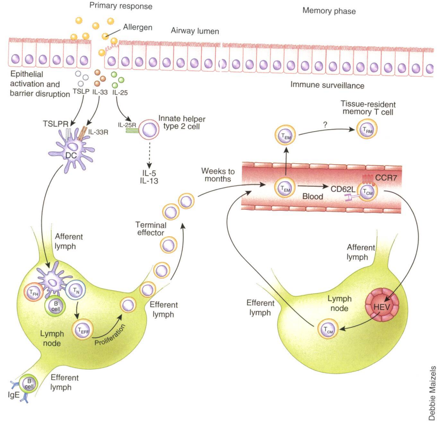

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**Figure 1** Generation of allergen-specific effector and memory T cells during primary immune responses at the epithelial barrier. During the primary immune response to allergens, disruption of the epithelial barrier leads to epithelial cell production of cytokines, such as TSLP, IL-25 and IL-33. Allergens and other inflammatory cues in the presence of TSLP, IL-25 and IL-33 induce dendritic cell (DC) maturation and trafficking to tissue draining lymph nodes (LN) where dendritic cells activate naive T ($T_N$) cells to differentiate into $T_H2$-type effector ($T_{EFF}$) cells. Allergen-specific $T_{EFF}$ cells undergo clonal proliferation in the lymph nodes, and $T_{EFF}$ cells exit the lymph nodes through efferent lymphatics. $T_{EFF}$ cells re-enter the systemic circulation through the thoracic duct and enter inflamed tissue through postcapillary venules. The majority of $T_{EFF}$ cells eventually die, but a minority differentiate into memory T ($T_M$) cells several weeks after the resolution of inflammation. Depending on the profile of trafficking receptors expressed, different subsets of $T_M$ cells perform distinct types of immune surveillance in the steady state. Naive T cells and central memory T ($T_{CM}$) cells equipped with L-selectin (CD62L) and the chemokine receptor CCR7 have restricted and specific access to secondary lymphoid tissue through specialized high endothelial venules (HEVs), which express their respective ligands (PNAd and CCL21). $T_{CM}$ cells recirculate between lymphoid tissue and blood. Effector memory T ($T_{EM}$) cells primarily recirculate between the blood and peripheral tissue. $T_{RM}$ cells persist in peripheral tissue and do not recirculate. TSLPR, TSLP receptor; IL-33R, IL-33 receptor.

T cells, some allergy-prone organs that are exposed to the environment 'program', or imprint, preferential tissue-specific inflammatory effector T cell and memory T cell entry from the circulation to streamline immune surveillance, as many microbes are tissue tropic in virulence[12,17,22].

T cells that are programmed to home to the small intestine are imprinted during the initial antigen priming to specifically express integrin $\alpha_4\beta_7$ and the chemokine receptor chemokine (C-C motif) receptor 9 (CCR9), expression of which enables restricted and regulated entry into the small intestine[23,24] (**Fig. 2**). In contrast, the signature-homing receptors of skin-tropic T cells are the lectin cutaneous leukocyte antigen (CLA), which is derived from the glycosylation of PSGL1, in combination with the chemokine receptors CCR4 and CCR10 and, for some T cell subsets, CCR8 and CCR6 (**Fig. 2**)[20,25–32]. Low levels of the ligands for these receptors are constitutively expressed in the steady state to facilitate tissue-specific memory T cell homing and immune surveillance[30,33–36]. Higher levels of the ligands induced during inflammation promote increased tissue-specific effector T cell homing. Notably, mechanisms that facilitate gut- and skin-specific imprinting are dependent on tissue-specific dendritic cells and tissue-specific environmental cues[12,22]. In the gut, CD103$^+$ ($\alpha_E\beta_7$ integrin) lamina propria and Peyer's patch dendritic cells produce high concentrations of retinoic acid; retinoic acid enables these dendritic cells to imprint expression of the gut-tropic receptors $\alpha_4\beta_7$ and CCR9 on naive T cells[37–42]. Likewise, skin dendritic cells can generate high concentrations of the active vitamin D3 metabolite, 1,25(OH)$_2$D3, and then use it to induce CCR10 expression on T cells in higher mammals[22,43].

Studies suggest that the lungs and nasal-associated lymphoid tissue (NALT) also program T cells for tissue-specific homing[12,44]. High endothelial venules in NALT express high amounts of peripheral node addressin (PNAd), the ligand for CD62L, the expression of which distinguishes NALT from other mucosal-associated tissues, such as Peyer's patches. Programmed allergen-specific T cell entry into NALT through interactions with PNAd promotes allergic rhinitis[44]. Though not definitive, lung-specific imprinting is suggested by human studies in which CD8$^+$ T cells specific for respiratory, but not systemic, viruses were selectively enriched in human lungs, analogous to the enrichment of CLA$^{hi}$ T cells specific for skin-tropic but not systemic viruses in the skin[45,46]. Steady-state human lung-resident T cells are also reported to be enriched for CCR5, CCR6, chemokine (C-X-C motif) receptor 3 (CXCR3) and the integrins VLA1 ($\alpha_1\beta_1$), $\alpha_E$ ($\alpha_E\beta_7$ or CD103) and VLA4 ($\alpha_4\beta_1$)[47–49]. However, the cellular mechanisms and environmental cues that promote lung-specific T cell imprinting have not been elucidated.

A consequence of naive T cell priming at any tissue site is the generation of memory T cells after the resolution of inflammation; recently a category of memory T cells known as tissue-resident memory T cells ($T_{RM}$) has been characterized[50] (**Fig. 1**). Based on functional traits that are linked to the ability to traffic to either secondary lymphoid tissue or peripheral tissue, memory T cells are classified into 'central memory' and 'peripheral memory' T cells[51] (**Fig. 1**). Central memory T cells preferentially traffic between secondary lymphoid tissue and the systemic circulation, whereas effector memory T cells preferentially home to peripheral tissue from the blood[14,15,51] (**Fig. 1**). A fraction of antigen-primed effector T cells differentiate into long-lived antigen-specific

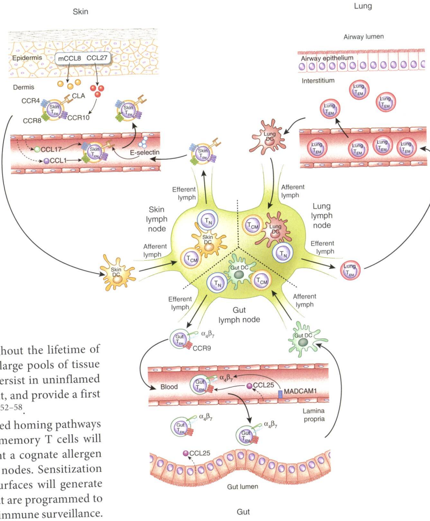
Skin
Lung
Epidermis
mCCL8 CCL27
Dermis
CCR4
CLA
Skin $T_{EM}$
CCR8
CCR10
Skin $T_{EM}$
CCL17
CCL1
Skin $T_{EM}$
E-selectin
Skin $T_{EM}$
Airway lumen
Airway epithelium
Interstitium
Lung $T_{EM}$
Lung $T_{EM}$
Lung $T_{EM}$
Lung DC
Lung $T_{EM}$
Lung $T_{EM}$
Lung $T_{EM}$
Lung $T_{EM}$
Efferent lymph
Afferent lymph
Skin lymph node
Lung lymph node
$T_N$
$T_{CM}$
Lung DC
Skin DC
Afferent lymph
Efferent lymph
$T_{CM}$
$T_N$
Skin DC
Gut DC
$T_N$
$T_{CM}$
Lung $T_{EM}$
Efferent lymph
Afferent lymph
Gut lymph node
Gut $T_{EM}$
$\alpha_4\beta_7$
CCR9
Gut DC
Blood
Gut $T_{EM}$
$\alpha_4\beta_7$
CCL25
MADCAM1
Lamina propria
Gut $T_{EM}$
$\alpha_4\beta_7$
Gut $T_{EM}$
$\alpha_4\beta_7$
CCL25
Gut lumen
Gut

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**Figure 2** Tissue-specific imprinting and steady-state programmed memory T cell trafficking to atopy-prone organs. Dendritic cells of the skin and the gut imprint T cells during the primary immune response to express trafficking receptors that enable tissue-specific homing. Skin T cells express CLA, CCR10, CCR4 and CCR8, which enable skin $T_{EM}$ cell entry into the skin in the steady state, as postcapillary venules in the skin express CCL17, E-selectin and CCL1 (the respective ligands for CCR4, CLA and CCR8) and epidermal cells expresses CCL27 (the ligand for CCR10) and mCCL8 (the ligand for CCR8 in the mouse). Gut $T_{EM}$ cells are programmed to express CCR9 and $\alpha_4\beta_7$, which enable the entry of gut $T_{EM}$ cells into the small intestine through postcapillary venules that express CCL25 and MADCAM1, which are the respective ligands for CCR9 and $\alpha_4\beta_7$. Skin- and gut-specific programmed lymphocyte homing pathways are well characterized in human and mouse studies. Human studies also suggest that programmed tissue-specific homing of lung $T_{EM}$ cells may also occur.

memory T cells, some of which persist throughout the lifetime of the host[50]. Recent studies have suggested that large pools of tissue $T_{RM}$ cells with enhanced effector capabilities persist in uninflamed peripheral organs, such as the lung, skin and gut, and provide a first line of defense against pathogens (**Fig. 1**)[32,48,50,52–58].

In the context of allergic diseases, programmed homing pathways increase the likelihood that tissue-patrolling memory T cells will encounter activated dendritic cells that present a cognate allergen in the tissue as well as in the draining lymph nodes. Sensitization to environmental allergens at the epithelial surfaces will generate long-lived allergen-specific memory T cells that are programmed to home to the mucosal and epithelial surfaces for immune surveillance. Concordantly, allergen-induced pathological activation of the epithelial barrier, through the production of cytokines such as IL-33, TSLP and IL-25, enhances the activation and maturation of $T_H2$-priming dendritic cells. Prior and recurrent allergen exposure will also generate higher frequencies of recirculating allergen-specific memory T cells, as well as long-lived allergen-specific $T_{RM}$ cells at the tissue sites of allergen exposure (**Fig. 1**). Thus, instead of being a very rare event, programmed homing pathways increase the likelihood that after allergen re-exposure, tissue-patrolling recirculating memory T cells and $T_{RM}$ cells encounter cognate allergen presented by dendritic cells, particularly if the epithelial barrier is compromised[59].

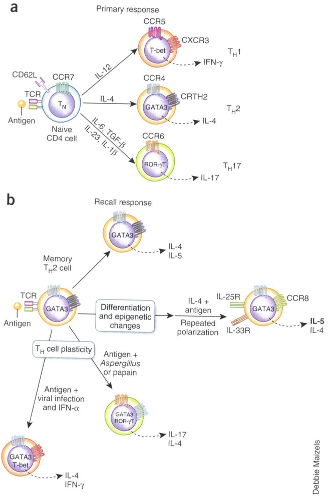
## Programmed regulatory T cell trafficking to the epithelial barrier

Programmed trafficking pathways that promote T cell–mediated allergic inflammation are also used by regulatory T ($T_{reg}$) cells to rein in inappropriate responses at mucosal and epithelial surfaces. The dendritic-cell–derived factors retinoic acid and $1,25(OH)_2D3$ can be either proinflammatory or anti-inflammatory[60–65]. In the intestine, retinoic acid derived from $CD103^+$ mucosal dendritic cells induces forkhead box P3 (Foxp3)$^+$ $T_{reg}$ cell differentiation from naive T cells after *in vivo* activation[63,65,66]. Retinoic acid also imprints $Foxp3^+$ adaptive $T_{reg}$ cells in the mesenteric lymph node (MLN) to acquire expression of $\alpha_4\beta_7$ and CCR9 to enable homing to the small intestine from the MLN[61]. In mice deficient for integrin $\beta_7$ or the integrin ligand mucosal vascular addressin cell adhesion molecule 1 (MADCAM1), oral tolerance is abrogated, as $T_{reg}$ cells fail to traffic from the MLN to the lamina propria[67]. In contrast, in the presence of pro-inflammatory cytokines, such as IL-15, retinoic acid can act as an adjuvant to promote inflammatory responses to dietary antigen[68]. In the skin, skin-tropic effector memory T cells and skin-tropic $T_{reg}$ cells share receptor profiles comprised of CCR4, CLA and CCR8, which suggests that common environmental cues may imprint proinflammatory and anti-inflammatory T cell homing pathways to the skin[13,69]. $T_{reg}$ cells exert their suppressive effects by localizing to lymphoid organs during the induction of the immune response and to peripheral tissues during the ongoing immune response[13,67,70–72]. For example, oral tolerance to ingested soluble antigen is initiated during the inductive phase of the immune response in the MLN, and oral tolerance is abrogated after mesenteric lymphadenectomy and in CCR7-deficient mice[71]. An altered epithelial barrier may allow proinflammatory cytokines or persistent allergen exposure to tip a programmed tissue-tropic pathway that promotes tolerance to innocuous allergens toward one that induces allergic inflammation.

## Tissue-resident memory T cells and allergic disease

A convincing body of work over the past decade has shown that antigen-experienced effector memory CD4 and CD8 T cells persist for long periods of time in uninflamed peripheral tissue as functional $T_{RM}$ cells[32,48,50,52–58]. Steady-state migration of pathogen-specific

**Figure 3** Chemokine receptors associated with CD4$^+$ helper T cell subsets. (**a**) During the primary immune response, cytokines (indicated above the arrow) and transcription factors (T-bet, GATA3 and ROR-γT) instruct naive T cells to differentiate into $T_H$ cell subsets. After TCR activation, specific cytokines derived from the innate immune system and unique master transcription factors direct naive CD4 $T_H$ cells to differentiate into $T_H1$, $T_H2$, $T_H17$, $T_{reg}$ and $T_{FH}$ cell subsets. Differentiated $T_H$ cell subsets produce lineage-specific effector cytokines and express characteristic chemokine receptors. (**b**) After recall stimulation with an antigen, allergen-specific memory $T_H2$ cells rapidly produce higher amounts of effector cytokines, such as IL-4, on rechallenge with the cognate allergen as compared to naive T cells. Repeated allergen-induced $T_H2$ cell differentiation induces a highly differentiated, CCR8$^+$IL-5$^{hi}$ $T_H2$ cell subset that coexpresses the indicated receptors. Memory $T_H2$ cells may not be fixed in differentiation along the $T_H2$ lineage and may adopt features of $T_H17$ and $T_H1$ cells on rechallenge with allergen in the context of inflammatory cues derived from fungi or viruses, respectively. TGF-β, transforming growth factor β; IL-25R, IL-25 receptor.



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memory CD8 T cells from the circulation into the lung, skin and gut is more restricted compared to other peripheral organs[55,56]. In infectious models, memory CD8 T cell persistence is antigen dependent in the lung and is antigen independent in the skin and intestine[55,73]. Re-exposure to antigen can also increase the size of the $T_{RM}$ cell pool[57]. $T_{RM}$ CD4 and CD8 T cells can be activated by antigen-presenting dendritic cells in tissue before activation by dendritic cells in the draining lymph nodes[54,58,74]. In addition to a functional role for $T_{RM}$ cells in host immunity to pathogens, a functional role for $T_{RM}$ cells has been shown in chronic inflammatory disease[53,75]. Memory CD4 T cells derived from human psoriasis skin and grafted into mouse dermis are sufficient for induction of disease in a xenotransplanation model of psoriasis[75].

In humans, large reservoirs of $T_{RM}$ cells have been reported in the skin and lungs[32,48]. Twice as many $T_{RM}$ cells are present in healthy skin compared to blood, and 98% of all CLA$^+$ T cells are found in the skin in the steady state[32]. Likewise, steady-state $T_{RM}$ cells in the human lung are equivalent in number to the circulating steady-state memory T cells in the blood[48]. Consistent with *in vivo* mouse studies, these lung $T_{RM}$ cells are not anergic, and they produce effector cytokines after activation[48]. Moreover, only lung $T_{RM}$ cells robustly proliferated *ex vivo* in response to influenza virus, a respiratory pathogen, in an antigen-specific manner, whereas skin-resident and blood T cells did not[48].

It is unclear whether and how $T_{RM}$ cells modulate allergic inflammation. The traditional thinking has been that the hugely expanded pool of allergen-specific effector T cells derived from dendritic-cell–activated central memory T cells express inflammatory chemokine receptors that enable their exit from lymphoid organs and their entry into peripheral sites of inflammation from the circulation[76]. Although the majority of effector T cells in a recall response are probably derived from central memory T cells, this paradigm may require revision with the appreciation that pre-existing tissue pools of $T_{RM}$ cells can be activated by tissue-resident dendritic cells. $T_{RM}$ cells may locally modulate allergic responses during the initiation phase of an allergen-specific recall response within the first few days after exposure, before lymph-node–derived amplified allergen-specific effector T cell entry into inflamed tissue from the bloodstream.

## Inflammatory $T_H2$ cell trafficking

CD4 $T_H$ cells have a pivotal role in mediating adaptive immunity to diverse pathogens and in promoting autoimmune diseases and allergic inflammation[77]. During inflammation and after T cell receptor (TCR) activation, specific cytokines derived from the innate immune system and distinct master transcription factors program naive $T_H$ cells to differentiate into $T_H1$, $T_H2$, $T_H17$, $T_{reg}$ and follicular T helper ($T_{FH}$) cell subsets[77]. The master transcriptional regulators T-box 21 (T-bet), GATA binding protein 3 (GATA3), RAR-related orphan receptor γ (ROR-γT), FOXP3 and B cell CLL/lymphoma 6 (BCL6) drive $T_H$ cell subset differentiation and impart specificity of $T_H$ cell lineage function through the production of signature effector cytokines[77] (**Fig. 3**). Repeated antigen exposure and progressive lineage polarization also promote terminal $T_H1$ and $T_H2$ cell differentiation through epigenetic modifications at the *Ifng* and *Il4* loci to enable rapid effector cytokine production after antigen rechallenge[78]. Polarizing cytokines and master transcription factors that promote $T_H1$, $T_H2$ and $T_H17$ cell lineage differentiation also program the expression of unique combinations of inflammation-specific chemokine receptors on $T_H$ cell lineages during priming[13,15,79] (**Fig. 3**). Thus, $T_H2$ cells are programmed during lineage differentiation to selectively express the chemokine receptors CCR4, CCR8 and, in humans, the prostaglandin D2 chemoattractant receptor DP2 or CRTH2 (refs. 79–81), which are associated with $T_H2$-type inflammation.

## $T_H2$ cell heterogeneity

$T_H2$ cells can also be divided into an IL-4$^{hi}$IL-5$^{lo}$ subset and a more differentiated IL-4$^{lo}$IL-5$^{hi}$ subset, which can be distinguished by their expression of CCR4 or CCR8, respectively[29,82]. Upregulation of CCR4 expression on $T_H2$ cells occurs rapidly after TCR ligation irrespective of prior antigen exposure and the extent of $T_H2$ differentiation[29,83]. However, CCR8 expression and robust expression of IL-5 in $T_H2$ cells is restricted to more terminally differentiated $T_H2$ cells that have previously been exposed to antigen[29,69,82,84,85]. Other examples of lineage-specific gene expression in more terminally differentiated T helper subsets include the IL-33 receptor (also called T1ST2 or ST2) in more terminally differentiated $T_H2$ cells and hepatitis A virus cellular receptor 2 (TIM3) in highly differentiated $T_H1$ cells[86,87].

Although it was previously believed that highly differentiated T helper subsets are fixed with regard to lineage differentiation, plasticity in $T_H17$, $T_{reg}$, $T_{FH}$ and $T_H2$ cell lineage differentiation is now appreciated, as is the fact that additional $T_H2$ cell subsets exist *in vivo* during chronic inflammation[88–90]. Virus infection and the induction of interferons reprogram antigen-specific, IL-4–producing GATA3$^+$ $T_H2$ memory cells to become $T_H2+1$ cells that coexpress GATA3 and T-bet and secrete both IL-4 and interferon γ (IFN-γ)[88]. Likewise, $T_H2$ memory cells differentiate into a subset of IL-17–producing $T_H2$ cells that coexpress GATA3 and ROR-γT in severe chronic allergic inflammation that manifests a robust mixed neutrophilic and eosinophilic infiltrate *in vivo*[89].

This heterogeneity of $T_H2$ cells should be kept in mind, as the original studies that elucidated $T_H$ cell subset biology were primarily conducted in tissue culture in the presence of neutralizing antibodies to generate pure $T_H$ cell lineages. During chronic inflammation in the absence of neutralizing antibodies *in vivo* and in the setting of genetic and environmental heterogeneity, human allergic disease is more complex and involves the simultaneous production of multiple cytokines. This is concordant with the current appreciation that human asthma is a heterogeneous disease and that therapeutic strategies that target a single $T_H2$ cell cytokine may only be effective in subpopulations of patients with asthma in which the therapeutic intervention is matched to the underlying pathophysiology[91,92]. Thus, *ex vivo* studies of human peripheral blood T cells have not provided a clear cut correlate of $T_H1$ and $T_H2$ effector cell cytokine production and the signature chemokine receptors that are associated with pure $T_H1$ and $T_H2$ lineage-differentiated cells[16,93]. It therefore follows that T cell–mediated allergic disease probably involves multiple chemokines and their receptors, the relative roles of which will vary depending on the affected organ and the temporal phase of the disease. We illustrate these points in the following sections.

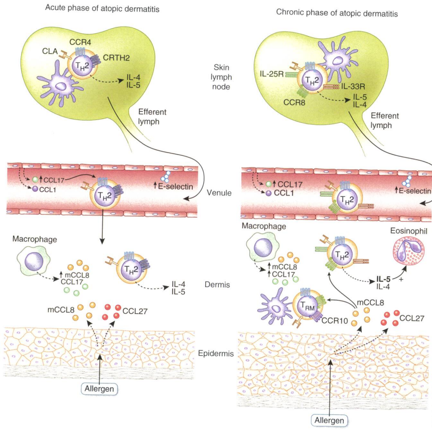
## T lymphocyte trafficking in atopic disease

**Atopic dermatitis.** The role of CD4$^+$ T cell effector functions in the pathogenesis of atopic dermatitis has been established in both mouse and human studies[94,95]. In human atopic dermatitis, a number of skin-homing trafficking receptors, such as CLA, CCR4, CCR10 and CCR8, as well as their ligands, have been implicated and reported to correlate with disease activity[94,96–99]. Although the contributions of CCL17 and CCL27 in the recruitment of human memory CD4 T cells from the blood to the sites of skin inflammation are well established, a correlation of skin CCL17 and CCL27 transcripts with specific inflammatory human disease phenotypes has not been found[100]. In contrast, comparative analyses of all known chemokine RNA transcripts in healthy skin from control individuals and lesional skin from individuals with different chronic inflammatory skin disorders found significant and specific induction of the chemokines CCL1 and CCL18 in inflamed atopic dermatitis skin[34,101]. Currently, the receptor for CCL18 is not known, although CCL18 binds skin-tropic CLA$^+$ memory T cells in patients with atopic dermatitis and induces *in vivo* migration of human memory T cells[102]. Additionally, microarray analyses have revealed that expression of the enzyme fucosyltransferase VII (FucT-VII), which regulates CLA expression, as well as the chemokine receptors CCR4 and CCR10, was significantly elevated in blood CD4$^+$ T cells from individuals with atopic dermatitis compared to healthy controls[103,104]. Notably, the frequency of CLA$^+$ blood T cells correlates with disease activity and skin inflammation in both contact hypersensitivity and atopic dermatitis[105]. Additional studies have suggested a role for chemokine (C-X3-C) receptor 1 (CX3CR1) and its ligand, CX3CL1, in active atopic dermatitis and are concordant with studies that suggest a role for CX3CR1 in asthma[106–108].

The *in vivo* role of trafficking receptors that regulate T cell–mediated atopic skin inflammation has recently been clarified in two complementary studies that used a 7-week treatment schedule in a mouse model of chronic atopic dermatitis[109]. CCL27 promotes atopic dermatitis in this model[28]. Recent studies highlighted how the kinetics of chemokine and chemokine-receptor expression, tissue-specific T cell trafficking and tissue reservoirs of antigen-specific T cells might regulate cutaneous inflammation during acute, recurrent or chronic allergen exposure (**Fig. 4**)[29,110]. CCR4 is essential for the acute entry of T cells into the skin through the cutaneous vasculature and drives atopic inflammation in antigen-naive skin, in which pre-existing skin-resident reservoirs of antigen-specific memory T cells are absent[110]. Thus CCR4 is crucial for T cell entry into the skin during the initiation phase of cutaneous allergic inflammation, which is concordant with the rapid kinetics of the induction of its ligand, CCL17, in skin endothelium and with findings in other models of acute cutaneous inflammation[25,111]. However, after repeated cutaneous allergen sensitization, CCR4 is not necessary for the entry of $T_H2$ cells from cutaneous vascular beds into the skin to sustain chronic eosinophilic atopic inflammation[29]. In this setting, pre-existing skin pools of allergen-specific memory $T_H2$ cells are probably sufficient to initiate and drive local inflammatory responses through effector cytokine production after activation by skin-resident antigen-presenting cells. In contrast, in this setting, both CCR8 and its newly described second ligand, mCCL8 (a ligand for CCR8 in the mouse), are essential for promoting IL-5–driven eosinophilic inflammation in chronic atopic dermatitis[29]. After 7 weeks of treatment, when peak atopic inflammation is manifest, mCCL8 is the only T cell–attracting chemokine that is significantly induced in sensitized skin, and its expression is several folds higher than those of CCL1, CCL17 and CCL22. Moreover, trafficking of CCR8$^+$ $T_H2$ cells, which are enriched for IL-5 and the IL-25 receptor, into allergen sensitized skin is essential for driving skin inflammation, as both IL-5 and IL-25 are cytokines that amplify eosinophilic inflammation (**Fig. 4**)[5,112].

**Asthma.** The mechanisms of T cell–mediated inflammation have been extensively characterized in human asthma and in mouse models of allergic pulmonary inflammation and have identified CD4$^+$ $T_H2$ cells as being central to the pathogenesis of human asthma. This concept has been established through studies of the specific effects of $T_H2$ cytokines and the *in vivo* findings that $T_H2$ cells are sufficient to initiate and drive asthma pathogenesis[59,77,113,114]. However, it is now well appreciated that other T cell subtypes, such as $T_H1$, $T_H17$, $T_{reg}$, natural killer T (NKT) and innate helper type 2 cells coexist with $T_H2$ cells and contribute to the pathogenesis of asthma[113,114]. Furthermore, the plasticity of $T_H2$ lineage differentiation, such that $T_H2$ cells may undergo additional differentiation to coproduce IFN-γ or IL-17, has been shown in mouse models of allergic pulmonary inflammation and, for IL-17, in human asthma[88,89]. Given the chronicity of human asthma, the possibility that various environmental

**Figure 4** Trafficking of $T_H2$ cell subsets into the skin during the acute and chronic phases of atopic dermatitis. During the acute phase of atopic dermatitis, the initiation of inflammation is dependent on $T_H2$ cell entry into inflamed skin by CCR4 and the CCR4 ligand CCL17, which is upregulated in inflamed postcapillary venules. During the chronic phase of atopic dermatitis, the pool of allergen-specific skin tissue $T_{RM}$ cells is increased as compared to in the acute phase, and these allergen-specific $T_{RM}$ cells can initiate inflammation independent of CCR4-dependent $T_H2$ cell entry from the blood. Repeated prior allergen exposure also leads to the *in vivo* generation of the more differentiated CCR8$^+$IL-5$^{hi}$ $T_H2$ cell subset in the chronic phase. Allergen challenge induces production of the CCR8 ligand, mCCL8, in the skin. mCCL8-dependent trafficking of IL-5$^{hi}$ CCR8$^+$ $T_H2$ cells into the skin drives eosinophilic skin inflammation in the chronic phase of atopic dermatitis in the mouse model.



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exposures that induce recurrences and exacerbations throughout the course of disease cumulatively modulate the phenotype of an individual's allergic inflammation is not surprising. For example, respiratory viral infections and environmental mold exposure have been shown to be key variables in the exacerbation of allergic asthma[115–118].

The clinical presentation of asthma led us to focus our efforts on dissecting the mechanisms that underlie T cell trafficking and inflammation in the lung in the context of the asthmatic response, which can be divided into an early and a late phase[119]. The early phase of the asthmatic response occurs rapidly (within 5–60 min of allergen exposure) as a result of mast cell degranulation and mediator release and is characterized by airway swelling and bronchoconstriction, which results in a corollary decrease in airway airflow (**Fig. 5**)[119]. The early phase response often resolves but is followed several hours (>4) later by a more severe late phase response, which is induced by the infiltration of inflammatory cells, such as T cells and eosinophils in the airways, and is also associated with reduced airway airflow[119]. The late phase asthmatic response can last for extended periods of time[120]. Moreover, recurrent and constant exposure to inciting allergens drives inflammation and permanent airway remodeling in individuals with chronic asthma[119]. We first showed that T cell trafficking is necessary for asthmatic inflammation by establishing the essential requirement of GPCR signaling in allergic airway inflammation, for which we used an adoptive transfer model of asthma[121,122]. T cell trafficking during the early and late phase asthmatic response involves multiple trafficking receptors, which are regulated by both innate and adaptive pathways and are summarized below and reviewed in more detail in ref. 123 (**Fig. 5**).

Airway mast cells are the innate cells that are crucial for the early phase recruitment of T cells and initiation of the asthmatic response. When sensitized individuals inhale an allergen, pre-existing allergen-specific immunoglobulin E (IgE) leads to the crosslinking of mast cell IgE Fc receptors and the release of $T_H2$-type effector cytokines, inflammatory mediators, leukotrienes, prostaglandins and inflammatory chemokines by mast cells into the airways (**Fig. 5**). Among the leukotrienes released by mast cells, leukotriene $B_4$ ($LTB_4$) is rapidly generated within minutes of exposure from membrane lipids and is the lipid chemoattractant ligand for the GPCR leukotriene $B_4$ receptor 1 (BLT1), which is expressed by $T_H1$, $T_H2$ and $T_H17$ cells and induces their migration (unpublished observations and ref. 124). A deficiency of BLT1 leads to a defect in early T cell accumulation in the airways in a model of allergic pulmonary inflammation that involves active allergen immunization and the generation of allergen-specific IgE[124,125]. Mast cells also produce the chemokines CCL1, CCL17 and CCL22 (refs. 34,126,127). Mast cell release of CCL1 may be key for early $T_H2$ cell recruitment through the CCR8 receptor, as was shown in a mast-cell–dependent model of chronic allergic inflammation[126]. The prostaglandins released include prostaglandin D2 (PGD2), the receptors for which are the GPCRs DP1 and DP2. DP2 is also called CRTH2 (chemoattractant receptor-homologous molecule expressed on $T_H2$ cells) and is expressed by human $T_H2$ cells. PGD2 induces migration and activation of $T_H2$ cells through CRTH2, and polymorphisms of CRTH2 are associated with an increased risk for asthma in Chinese children and African Americans but a reduced risk for asthma in German children[81,128,129]. The rapid kinetics of mast cell–mediator release suggests that the recruitment of T cells in this early phase probably involves lung-resident allergen-specific memory cells.

During the late, T cell–mediated effector phase of asthma, initial T cell recruitment is probably facilitated by allergen- and damage-associated molecular pattern-mediated activation of pattern recognition receptors, such as Toll-like receptors (TLRs) or C-type lectins on innate immune cells, or by direct activation of epithelial cells by allergens. This induces the production of $T_H2$-promoting cytokines and chemokines by epithelial cells and innate tissue leukocytes and an initial phase of STAT6-independent recruitment of a small number of allergen-specific T cells into the lungs[121]. The kinetics of T cell recruitment in this initial phase suggests that it may also involve lung-resident allergen-specific memory and effector cells. For example, TLR activation and

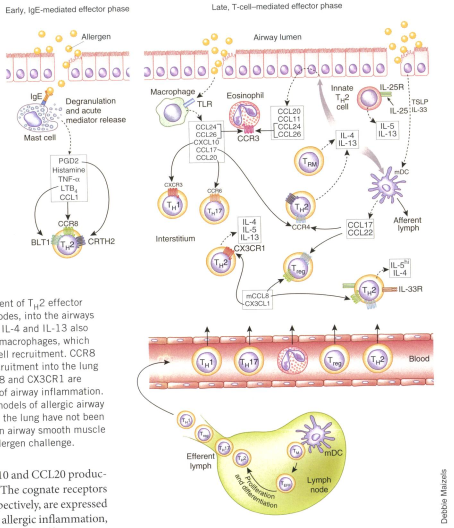

**Figure 5** Chemokine-regulated $T_H$ cell trafficking into the lung during the early and late phases of the asthmatic response. During the early phase allergic airway response (left), allergen-IgE complexes stimulate airway mast cells to degranulate and release acute mediators such as histamine, tumor necrosis factor α (TNF-α), $LTB_4$, PGD2 and the chemokine CCL1. $LTB_4$, PGD2 and CCL1 recruit $T_H2$ cells into the airways through BLT1, CRTH2 and CCR8, respectively. T lymphocyte trafficking during the late phase allergic airway response (right) is facilitated by innate cells, such as macrophages and myeloid dendritic cells (mDCs). Allergen-induced TLR activation induces macrophage and epithelial cell release of chemokines such as CXCL10 and CCL20. These chemokines recruit an initial wave of T cells into the airways through their receptors, CXCR3 and CCR6. IL-4 and IL-13 produced by activated lung allergen-specific $T_{RM}$ cells stimulate mDCs to release the chemokines CCL17 and CCL22 in a STAT6-dependent fashion. These chemokines amplify the subsequent recruitment of $T_H2$ effector cells, which are generated in lung draining lymph nodes, into the airways from the circulation by the $T_H2$ cell receptor CCR4. IL-4 and IL-13 also induce the differentiation of alternatively activated macrophages, which produce CCL17 to promote CCR4-dependent $T_H2$ cell recruitment. CCR8 and CX3CR1 are also probably involved in T cell recruitment into the lung during the late phase allergic airway response. CCR8 and CX3CR1 are implicated in human asthma and in mouse models of airway inflammation. The CCR8 ligand mCCL8 is upregulated in mouse models of allergic airway inflammation, but the cellular sources of mCCL8 in the lung have not been defined. The CX3CR1 ligand, CX3CL1, is induced in airway smooth muscle cells, lung endothelium and epithelial cells after allergen challenge.

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allergen exposure are reported to induce CXCL10 and CCL20 production by macrophages and epithelial cells[123,130]. The cognate receptors of CXCL10 and CCL20, CXCR3 and CCR6, respectively, are expressed by human T cells that infiltrate the lungs during allergic inflammation, particularly the airways 24 h after allergen challenge[49,131,132]. Moreover *Ccr6*$^{-/-}$ mice have diminished airway hyper-responsiveness and allergic inflammation, and adoptive transfer of *Ccr6*$^{+/+}$ T cells into *Ccr6*$^{-/-}$ mice increases allergic lung inflammation[133,134]. Additionally, tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) enhances the production of CCL20 by epithelial cells, perpetuating allergic inflammation through increased recruitment of CCR6$^+$ memory and effector $T_H2$ cells into the airways[135]. Likewise, findings with *Cxcl10*$^{-/-}$ and CXCL10 transgenic mice suggest that CXCR3$^+$ T cell recruitment enhances allergic pulmonary inflammation[136].

After the initial trafficking of T cells into the airways, $T_H2$ cell recruitment into the lungs is markedly amplified after T cell activation by mature dendritic cells in the draining lymph node, where T cells also undergo rapid proliferation[76]. The chemokine receptors CCR4, CCR8 and, possibly, CX3CR1 are the likely key chemokine receptors that regulate $T_H2$ cell recruitment into the lung during this phase. Concentrations of CCR4, CCR8 and their ligands are elevated in the lung or the bronchoalveolar lavage fluid of patients with asthma and in mouse models after allergen challenge[137–140]. However, deletion of CCR4 and CCR8 receptors in mouse models of asthma and in human studies has yielded conflicting results[126,141–146]. Thus, aside from in two studies[126,141], CCR8 has not been shown to drive $T_H2$-type effector cell recruitment in models of allergic airway inflammation[126,141,143,145,146]. Likewise, 24 h after allergen challenge of steroid-naive subjects with mild asthma, there was no difference in the expression of CCR4 or its ligands in the bronchoalveolar lavage fluid, although CCR4 was required for efficient tissue entry of $T_H2$ cells in an adoptive transfer model of acute allergic pulmonary inflammation[49,146]. In a recent study, CCR4$^+$ T cells in the peripheral blood positively correlated with asthma severity in a cohort that included subjects with mild asthma as well as steroid-dependent subjects with moderate to severe asthma, and *ex vivo* production of CCL17 was higher in bronchial biopsy explants from subjects with asthma compared to healthy controls[147]. Additionally, studies of airway allergen challenge and genome-wide association have implicated CX3CR1 and its ligand CX3CL1 in human asthma[108,148]. A recent study provided a possible mechanism for this association by showing that CX3CR1 signaling aided $T_H2$ cell survival and maintenance to drive allergic pulmonary inflammation[107]. Facilitating the long-term persistence of allergen-specific memory and effector T cells is also a mechanism by which the CCR8 pathway may promote chronic allergic inflammation[149].

To define the transcriptional signals that regulate the induction of inflammatory chemokines and amplify T cell recruitment into the asthmatic lung, we used an adoptive transfer model of acute allergic inflammation and showed that it is STAT6 dependent[121]. T cell recruitment was dependent on STAT6 expression by a resident lung cell, which we subsequently showed to be from a subset of CD11b$^+$CD11c$^+$ myeloid pulmonary dendritic cells, concordant with findings from other groups[150–152]. Thus, in this model of acute asthma, *Stat6*$^{-/-}$ mice had

**Table 1 Drugs in development or currently available that inhibit leukocyte migration**

| Target molecule | Target cell | Compound or drug | Company | Clinical indication and stage of development |
|---|---|---|---|---|
| CRTH2 (DP2) | T cell, eosinophil and basophil | QAW039, oral antagonist | Novartis | Moderate to severe asthma with sputum eosinophilia; phase 2, currently recruiting |
| | | ADC3680B, oral antagonist | Pulmagen | Partly controlled mild to moderate atopic asthma; phase 2 completed February 2012 |
| | | MK-7246, oral antagonist | Merck | In development |
| | | AZD1981, oral antagonist | AstraZeneca | In development |
| | | OC000459, oral antagonist | Oxagen | In development |
| | | AM156, topical antagonist | Amira | Allergic conjunctivitis; in development |
| | | Antagonist | Pfizer/Wyeth | In development |
| | | Antagonist | Millenium | In development |
| CRTH2 and DP1 | T cell, eosinophil and basophil | AMG 853, oral antagonist | Amgen | Asthma; phase 2 completed March 2011 |
| | | AMG-009, oral antagonist | Amgen | Asthma and allergic rhinitis; phase 1 planned |
| CCR3 | Eosinophil | GW-766904, oral antagonist | GlaxoSmithKline | Mild to moderate asthmatics with sputum eosinophilia; phase 2, currently recruiting |
| CCR4 | T cell | KW-0761, intravenous antibody | Kyowa Hakko | Allergic rhinitis and healthy volunteers; phase 1 completed |
| | | | Kyowa Hakko | Adult T cell leukemia-lymphoma; phase 2 completed April 2011 |
| | | | Amgen | Non-oncology, asthma and inflammatory indications; in development |
| | | Antagonist | Astellas | In development |
| | | Antagonist | Bristol-Meyers Squibb | In development |
| CCR9 | T cell | GSK1605786A | ChemoCentryx | Crohn's Disease; phase 1 and 2 completed |
| | | Oral antagonist | GlaxoSmithKline | Crohn's Disease; phase 3 recruiting |
| LFA1 (CD11a-CD18; $\alpha_L\beta_2$ integrin) | T cell | Efalizumab, subcutaneous antibody | Genentech | US Food and Drug Administration (FDA) approved in 2003 for treatment of moderate to severe psoriasis. Voluntarily withdrawn in 2009 because of association with fatal progressive multifocal leukoencephalopathy (PML) |
| VLA-4 ($\alpha_4\beta_1$ integrin) | T cell | Natalizumab, intravenous antibody | Biogen Idec | FDA approved in 2004 for relapsing-remitting multiple sclerosis. FDA black box warning: increased risk of PML. FDA approved in 2008 for moderate to severe Crohn's disease |

significantly lower production of the $T_H2$ cell–attracting chemokines CCL17 and CCL22, as well as decreased induction of the eosinophil-attracting chemokines CCL11 and CCL24. Moreover, CCL17, CCL22 and CCL11 were produced in a STAT6-dependent fashion in a 4-week active immunization model of allergic pulmonary inflammation[153]. In contrast, the transcriptional signals that regulate the induction of CCR8 and CX3CR1 in the allergic lung are not as well defined. Lung mCCL8 induction was STAT6 independent in a chronic 4-week active immunization model[153]. With respect to other T helper subsets, it is notable that $T_{reg}$ cells express the chemokine receptors CCR4 and CCR8 and, thus, may be recruited by the release of CCL1 during the early phase asthmatic response and the release of CCL17, CCL22 and mCCL8 during the late phase asthmatic response[69].

**Food allergy.** In humans, allergen-specific $T_H2$ cells are essential for triggering non–IgE-mediated food allergies as well as maintaining type I IgE-mediated allergic responses. In allergic individuals, allergen-specific T cells in the blood proliferate more in response to food allergens compared to in non-allergic individuals, and human milk-specific T cells derived from the duodenum have a dominant $T_H2$-type cytokine secretion profile[154,155]. After food challenges, the duodena of symptomatic individuals contain increased numbers of IL-4$^+$CD4$^+$ T cells associated with IgE$^+$ cells that were mostly mast cells despite lacking food-allergen–specific serum IgE[156]. With regard to programmed organ-specific trafficking molecules, the integrin $\alpha_4\beta_7$ is selectively increased in lymphocytes stimulated with β-lactoglobulin in children allergic to cow's milk[157]. Peach-protein-allergen–specific T cell clones are also selectively enriched for $\alpha_4\beta_7$ and lack expression of CLA[158]. Thus food-allergen–specific T cells in humans are imprinted with gut-specific homing receptors. Milk-induced skin urticaria is also a common manifestation of milk allergy in young children, and milk exposure induces symptoms of eczema in individuals with atopic dermatitis[159,160]. Notably, milk-induced urticaria and atopic eczema are both associated with increased circulating concentrations of allergen-specific T lymphocytes, which express CLA[159,160]. Additionally, a recent human study found that anaphylactic peanut allergy is associated with an increased frequency in the number of blood IL-5$^-$IL-4$^+$ peanut-specific $T_H2$ cells and IgE responses[161]. Conversely, blood IL-5$^+$IL-4$^+$ peanut-specific $T_H2$ cells are associated with IgE-independent allergic eosinophilic gastroenteritis[161]. Extending these observations, the majority of peanut-allergen–specific blood CD4 T cells express CCR4 in individuals with a history of peanut anaphylaxis and peanut-specific IgE, and peanut-allergen–specific cell lines from these individuals primarily produce IL-4 (ref. 162).

In the mouse, CCR7 expression on $T_{reg}$ cells is crucial for the induction of oral tolerance in the MLN, and $T_{reg}$ cells are directed to home to the gut lamina propria by MADCAM1 (refs. 67,71). In a mast-cell–dependent mouse model of chronic food allergy in which inflammation is induced in the small intestine and manifests as allergic diarrhea, allergen-specific T cells are induced in the MLN and traffic to the small intestine[163]. In this model, increased numbers of $T_H2$ cytokines and the CCR8 and CCR4 ligands CCL1, CCL17 and CCL22 are upregulated in the inflamed small intestine[163]. In this same model, CCR6 is crucial for T cell trafficking to the small intestine and for driving

IgE-independent $T_H2$ cell inflammation and allergic diarrhea[164]. Thus, chemokine receptors associated with allergic airway inflammation also seem to drive allergic inflammation in the gut.

## Therapeutic targeting of leukocyte trafficking in allergy

We suggest that programmed tissue-specific T cell trafficking pathways during immune surveillance and inflammation contribute to the propensity of the skin, gut and lung for developing allergic diseases. Blocking tissue- and inflammation-specific trafficking pathways provides an opportunity for developing new targeted therapies for inflammation and allergic diseases and is actively being pursued (**Table 1**). Integrins were the first category of trafficking receptors to be targeted for therapy. Efalizumab, an antibody to CD11a that inhibits skin-homing $CLA^+$ T cell entry into the skin, was the first migration-inhibitory drug to be approved by the US Food and Drug Administration, and although it was initially approved for the treatment of psoriasis, it was also reported to be effective in some individuals with atopic dermatitis[165]. Among the chemoattractant receptors, the first drug to successfully reach phase 3 clinical trials for an inflammatory disease was, in fact, an antagonist for another tissue-specific homing molecule, CCR9, for the treatment of Crohn's disease. With regard to allergic inflammation, based on population-based genetic studies of an association of CRTH2 polymorphisms with allergic disease, many companies are actively pursuing inhibitors of CRTH2, some of which have recently completed phase 2 trials[81,128,129,166]. Other chemokine receptors being targeted for anti-allergic therapy are CCR3 and CCR4. An advantage of anti-migration–based therapy is that the likelihood of toxicity is less than that of immunosuppressive therapy. However, a caveat of extended anti-integrin–based therapies has been an unpredicted increased risk for developing fatal progressive multifocal leukoencephalopathy[167].

In developing therapies based on anti-chemokine receptors, as we highlight in this review, the kinetics of inflammation-driven chemokine induction and T cell chemokine receptor expression during chronic allergic disease dictates the relative roles of different ligand-receptor pairs during distinct phases of disease and in different compartments. Large pre-existing pools of tissue-resident allergen-specific memory T cell pools also probably influence migration-inhibitor–based therapies. Though classical $T_H2$ cells are the main orchestrators of allergic inflammation through the production of $T_H2$ effector cytokines, other T helper subsets contribute to allergic inflammation. These factors reflect the importance of multiple T cell trafficking receptor pathways in allergic inflammation. Thus, the strategic design of new therapeutic agents directed against trafficking-receptor pathways for the treatment of allergic inflammation requires an appreciation of these multiple factors.

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# Asthma phenotypes: the evolution from clinical to molecular approaches

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**Although asthma has been considered as a single disease for years, recent studies have increasingly focused on its heterogeneity. The characterization of this heterogeneity has promoted the concept that asthma consists of multiple phenotypes or consistent groupings of characteristics. Asthma phenotypes were initially focused on combinations of clinical characteristics, but they are now evolving to link biology to phenotype, often through a statistically based process. Ongoing studies of large-scale, molecularly and genetically focused and extensively clinically characterized cohorts of asthma should enhance our ability to molecularly understand these phenotypes and lead to more targeted and personalized approaches to asthma therapy.**

## The evolving definition of asthma

Asthma affects 5–10% of the population in many developed countries and is associated with a large socioeconomic burden. Yet 'asthma' is a vague term that describes a group of clinical symptoms with reversible expiratory airflow limitation or bronchial hyperresponsiveness. Although international asthma guidelines have added "in the presence of airway inflammation" to the list of criteria for disease, inflammation is almost never measured in practice, and a consistent inflammatory process is rarely confirmed. Thus, the term asthma, like 'arthritis', equates to a definition of grouped clinical and physiological characteristics (**Fig. 1**). These characteristics could identify syndromes, phenotypes or even multiple diseases rather than a single disease. Even leading international clinical journals such as *The Lancet* have suggested that the term asthma is out of date and that the evolution of more detailed clinically and biologically focused definitions of this condition should be encouraged[1]. Yet, most mechanistic studies of asthma focus only on a highly specific process related to allergic airway inflammation, despite the fact that the overall importance of this single process to human disease remains poorly understood.

In the 1990s and early 2000s, this vague clinical definition of asthma led to successful clinical trials of nonspecific anti-inflammatory and bronchodilator medications. At the same time, researchers working with mouse models of allergic asthma and/or inflammation identified the crucial role of T helper ($T_H2$) immune pathway elements (**Fig. 2**) in both inflammation and airway hyperresponsiveness[2–4]. Thus, asthma was widely believed to be an allergic, eosinophilic and $T_H2$-mediated (and corticosteroid-responsive) disease[5–7]. Unfortunately, negative initial results from $T_H2$-focused human clinical trials virtually halted biological approaches to treating asthma.

Simultaneously, a subgroup of people with severe asthma were observed to have refractory disease in the absence of eosinophils, with further studies suggesting that responses of people with asthma to nonspecific anti-inflammatory drugs, such as inhaled corticosteroids, were dependent on the presence and type of airway inflammation[8–11]. It was then shown that an antibody to the allergy-related factor IgE showed efficacy in reducing exacerbations in a targeted population with 'allergic' asthma[12,13]. Thus, 'asthma' began to evolve from a term describing a single disease to one encompassing multiple subgroups or, as they are now termed, phenotypes[14,15].

## Definition of 'phenotype'

A phenotype is defined as the "observable properties of an organism that are produced by the interactions of the genotype and the environment"[16]. The concept of the phenotype have been suggested to be the prelude to that of the 'endotype', wherein a specific biological pathway is identified that explains the observable properties of a phenotype[17,18]. Although several endotypes of asthma have been proposed, none has been widely agreed upon; the acceptance even of asthma phenotypes is evolving, and the topic is controversial. Despite these difficulties in agreeing on endotypes, asthma phenotypes based on clinical characteristics, triggers or general inflammatory processes have been proposed, but there have been few attempts to link all of these characteristics together to better define phenotypes[19]. The definition of a true phenotype (or endotype) requires a unifying and consistent natural history, consistent clinical and physiological characteristics, an underlying pathobiology with identifiable biomarkers and genetics and a predictable response to general and specific therapies[18] (**Table 1**). Although both biased and unbiased approaches have begun to link the characteristics of asthma together to form phenotypes, no present system of subgrouping achieves all the requirements for a true phenotype or endotype. In addition, there are a number of co-morbidities and confounders that have been identified that can alter asthma phenotypes (**Box 1**).

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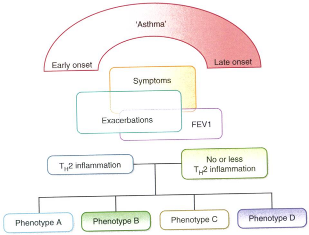

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**Figure 1** Schematic representation of the umbrella term 'asthma'. The key clinical features of severity (lung function, symptoms and exacerbations), inflammatory characteristics (particularly $T_H2$ immunity) and their division into associated phenotypes are shown. However, these phenotypes have not yet been fully characterized.

## Approaches to identifying phenotypes

**Biased approaches.** Asthma phenotyping began decades ago with the concepts of extrinsic (allergic) and intrinsic (nonallergic) asthma[14]. People with extrinsic asthma developed the disease early in life, were atopic (they made IgE specific to identifiable allergens) and had identifiable allergic triggers, other allergic diseases such as rhinitis or eczema or a family history of allergic disease. Intrinsic asthma developed later in life (after 40 years of age), was associated with aspirin-exacerbated respiratory disease (AERD) but not with allergic sensitization, and was generally not as well understood. Inflammatory biomarkers other than those related to IgE were not used. When small pathobiological studies in humans suggested that levels of $T_H2$ cytokines were similar in extrinsic and intrinsic asthma, and treatment with inhaled corticosteroids was found to be effective in the majority of mild to moderate asthma cases, the distinctions between extrinsic and intrinsic asthma fell out of favor[20–23].

Although most asthma is mild to moderate (and heterogeneity is indeed present in individuals with mild to moderate asthma), pathobiological studies in the 1990s of people with severe, refractory asthma reintroduced the concept of asthma heterogeneity with the finding that some of these individuals had neutrophilic inflammation that had not previously been reported in milder asthma[24]. Eosinophils were present in lung tissue from about 50% of people with severe asthma, and this group of individuals had a thicker subepithelial basement membrane (SBM), higher expression of transforming growth factor-β (TGF-β), more frequent and more severe symptoms, and more near-fatal events than individuals who had asthma without eosinophilic inflammation[8,25–27]. Age at onset (using age 12 as a cut-off) was reported to be an important factor in distinguishing atopic and allergic asthma from a less well-defined but more eosinophilic adult-onset phenotype[25]. Exercise-induced, obesity-related, smoking-related, neutrophilic and even 'paucigranulocytic' (the absence of an observable inflammatory process) were all suggested as asthma phenotypes, but few corresponding clinical and biological characteristics were identified.

**Unbiased approaches.** Because of concerns about clinical bias and the continued lack of specific cellular biomarkers for asthma phenotypes, several groups began to approach phenotyping in a manner that was less biased and more statistically based. Among these were a study in the United Kingdom that involved two groups of patients; a US study that involved a group, enhanced for individuals with severe asthma, from eight centers involved in the National Institutes of Health–sponsored Severe Asthma Research Program (SARP); and an analysis of two large European asthma cohorts[28–30].

The UK and SARP studies used cluster analysis, a method that combines various approaches to group important variables such as those related to asthma exacerbations, which include steroid bursts, emergency-room visits and hospitalization. These grouped variables were then used to produce clustered variables to identify phenotypes. However, the process of choosing variables introduces some bias. For instance, the UK cluster analysis included sputum eosinophils and bronchodilator responsiveness but did not include typical physiological measures of airway obstruction, such as forced expiratory volume in 1 second (FEV1); the SARP analysis included lung function but not inflammatory markers[28,29]. The European analysis was performed on people with asthma from across two European cohorts and used latent class analysis (LCA), which groups variables into latent classes under the statistically based assumption that the variables in a particular latent class are independent of each other. The European analysis had more limited phenotypic information than the other two studies.

Despite statistical variations in their approaches and the wide range of variables that were available and that were analyzed in each study, the results of the three studies are more similar than different, and they overlap with results obtained using the earlier, biased phenotype approaches. All three studies found age at disease onset to be a key differentiating factor. Early-onset disease is consistently associated with a more atopic and allergic condition over a range of severities, whereas later-onset disease is associated with eosinophilic inflammation and obesity, is more common in women and is generally less allergic. Interestingly, despite the association of early-onset disease with atopy and allergy, none of the unbiased approaches found variables associated with these conditions (such as atopy and total IgE) to be key distinguishing features of the subgroups.

A single cluster analysis has also been performed in children with asthma and was primarily limited to one urban center that included a large group of underserved children[31]. These children were predominantly atopic and allergic, as would be expected from a pediatric cohort, but greater severity was not correlated with greater numbers of skin-test reactions, higher IgE or higher exhaled nitric oxide (FeNO, a product of the $T_H2$-regulated inducible nitric oxide synthase (iNOS) enzyme), which generally tracks with atopy, allergy and the response to inhaled corticosteroids[32–34]. Rather, the determinants of severity were based primarily on asthma duration, medication use and lung function.

Thus, many of the elements needed for pathological and immunological definition of asthma phenotypes were missing from these studies, and only one cluster analysis was generally replicated in a second (albeit milder) cohort[28]. However, in addition to these clinically oriented clusters, a molecular phenotyping analysis in people with mild corticosteroid-naïve asthma has also been published[35]. In this study, the authors analyzed airway epithelial brushings for the expression of three genes upregulated by the $T_H2$-type cytokine interleukin-13 (IL-13) in epithelial cell cultures—*POSTN*, which encodes periostin; *CLCA1*, which encodes calcium-activated chloride channel regulator 1; and *SERPINB2*, which encodes serpin peptidase inhibitor, clade B, member 2—and used these signature genes to identify '$T_H2$-high' individuals. Of those analyzed, approximately 50% of people with

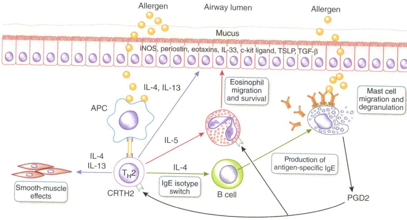
Allergen
Airway lumen
Allergen
Mucus
iNOS, periostin, eotaxins, IL-33, c-kit ligand, TSLP, TGF-β
IL-4, IL-13
Eosinophil migration and survival
Mast cell migration and degranulation
APC
IL-5
IL-4
IL-13
$T_H2$
IL-4
Production of antigen-specific IgE
Smooth-muscle effects
CRTH2
IgE isotype switch
B cell
PGD2

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**Figure 2** $T_H2$ immune processes in the airways of people with asthma. The pathway begins with the development of $T_H2$ cells and their production of the cytokines IL-4, IL-5 and IL-13. These cytokines stimulate allergic and eosinophilic inflammation as well as epithelial and smooth-muscle changes that contribute to asthma pathobiology. APC, antigen-presenting cell; CRTH2, chemoattractant receptor-homologous molecule expressed on $T_H2$ cells; iNOS, induced nitric oxide synthase; PGD2, prostaglandin D2; TSLP, thymic stromal lymphoprotein.

mild corticosteroid-naïve asthma had a $T_H2$-high signature in their airway epithelial tissue. Individuals who had asthma but did not have this signature had a similar gene-expression signature ($T_H2$-low) to that of healthy control subjects. Subjects classified as $T_H2$ high were subsequently found to have higher amounts of tissue IL-13 and IL-5 mRNA and greater numbers of eosinophils and mast cells, and they showed more atopy and SBM thickening compared to $T_H2$-low people[36]. Perhaps most importantly, these subjects responded to inhaled corticosteroid therapy, whereas the $T_H2$-low group did not, suggesting that this distinction may have profound clinical implications. Although clear patterns of clinical phenotype in relation to $T_H2$ gene expression have emerged from these studies, further long-term studies are needed to assess the stability of the identified phenotypes, integrate clinical clusters with biomarkers and, finally, identify responses to targeted therapies.

## $T_H2$-associated asthma

Almost since the inception of the concept that immunity can be divided into $T_H1$ and $T_H2$ type processes, asthma has been considered a $T_H2$ process that is linked strongly to atopy and allergy, type I hypersensitivity reactions, eosinophilic inflammation and response to corticosteroids. Indeed, data from biased and unbiased studies suggest that the majority of—but, clearly, not all—asthma cases fit this traditional view[28,29]. Current phenotyping approaches support the existence of an early-onset (usually during preadolescence), mostly atopic and allergic asthma phenotype, and most have additionally identified a later-onset (often age 20 or later) eosinophilic phenotype. The molecular and targeted therapy data support an overall $T_H2$ association with both of these phenotypes, such that these two clinically different yet immunologically overlapping phenotypes may fall into a broader category of $T_H2$-associated asthma (**Table 1** and **Fig. 3**). Finally, the clinical phenotype of exercise-induced asthma (EIA) is also likely to have a $T_H2$ component, given its eosinophil- and mast cell–related profile[37–39].

## Early-onset allergic $T_H2$ asthma

**Clinical and biological features.** Although a specific age cut-off for early-onset asthma has not been determined, most persistent adult asthma that originates in early childhood has an atopic and allergic component, and most people with asthma are likely to have this phenotype. However, the lack of responsiveness to corticosteroids and the lower concentrations of IgE in some children with asthma suggest that not all early-onset asthma is $T_H2$ associated[31,40].

Studies have suggested that age of onset is a better discriminator of adult asthma phenotypes than allergic factors, consistent with previous reports suggesting that allergic exposure and sensitization in childhood are only modestly associated with asthma development later in life[28,29,41]. Despite this, consistent relationships exist between allergic factors and onset of asthma in childhood. Early-onset asthma is typically associated with other atopic diseases, including allergic rhinitis and atopic dermatitis[14,25,28,29]; for example, 40% of people with early-onset asthma have a history of atopic dermatitis, whereas 4% of people with late-onset asthma do. The amounts of total and allergen-specific IgE are also higher in early-onset asthma than in later-onset asthma. People who have atopic asthma have higher

**Table 1 Asthma phenotypes in relation to characteristics**

| | Natural history | Clinical and physiological features | Pathobiology and biomarkers | Genetics | Response to therapy |
|---|---|---|---|---|---|
| Early-onset allergic | Early onset; mild to severe | Allergic symptoms and other diseases | Specific IgE; $T_H2$ cytokines; thick SBM | 17q12; $T_H2$-related genes | Corticosteroid-responsive; $T_H2$-targeted |
| Late-onset eosinophilic | Adult onset; often severe | Sinusitis; less allergic | Corticosteroid-refractory eosinophilia; IL-5 | | Responsive to antibody to IL-5 and cysteinyl leukotriene modifiers; corticosteroid-refractory |
| Exercise-induced | | Mild; intermittent with exercise | Mast-cell activation; $T_H2$ cytokines; cysteinyl leukotrienes | | Responsive to cysteinyl leukotriene modifiers, beta agonists and antibody to IL-9 |
| Obesity-related | Adult onset | Women are primarily affected; very symptomatic; airway hyperresponsiveness less clear | Lack of $T_H2$ biomarkers; oxidative stress | | Responsive to weight loss, antioxidants and possibly to hormonal therapy |
| Neutrophilic | | Low FEV1; more air trapping | Sputum neutrophilia; $T_H17$ pathways; IL-8 | | Possibly responsive to macrolide antibiotics |

## BOX 1 Comorbidities and confounders that alter asthma phenotypes

Numerous environmental factors including infection, smoking, hormonal factors, obesity (in $T_H2$ asthma) and others still to be identified can influence an underlying immunoinflammatory process. Although they are sometimes considered as separate phenotypes, these factors may have a more prominent role in altering the existing pathobiology. Innate immune pathways including Toll-like receptors, type II interferons and danger-associated molecular pathways in particular are likely to be important in nonallergic environmental exposures, which can both enhance and dampen $T_H2$ inflammation[138].

*History of smoking or exposure to second-hand smoke*[139]

- Worsens any existing asthma, probably through enhanced neutrophilic inflammation and oxidative stress
- Smoking-associated asthma may be a separate phenotype overlapping with chronic obstructive pulmonary disease

*Hormonal influences*[140]

- Associated with asthma initiation during times of hormonal changes (such as menarche, pregnancy or menopause), perhaps through enhanced $T_H2$ activation
- Involved in differences in asthma incidence and prevalence between sexes during adolescence
- May worsen or improve existing asthma during pregnancy
- Associated with asthma exacerbations before menses

*Viruses and bacteria*[141,142]

- Initiate and/or increase likelihood of developing $T_H2$-related asthma in atopic children
- Exacerbate existing asthma, possibly through type II interferon mechanisms
- May initiate adult-onset asthma in susceptible individuals in conjunction with atypical bacteria

*Occupational exposures*[143]

- Initiate asthma in susceptible hosts
- Worsen existing asthma

amounts of $T_H2$ cytokines and greater numbers of cells with receptor-bound IgE than people who have atopy but do not have asthma[20,21]. Within this group of asthma, the recent molecular distinction of mild $T_H2$-allergic asthma—which is associated with higher levels of eosinophils, mast cells, total and specific IgE as measured by allergy skin testing (atopy) and $T_H2$ cytokines than another subset of people who have mild asthma without evidence for $T_H2$ inflammation—supports these earlier studies and probably explains some of the variability in phenotypes observed earlier[35].

Early-onset allergic asthma can present with mild to severe disease, but it is unclear whether mild allergic asthma progresses to severe disease or whether severe allergic asthma arises in childhood and remains severe[31,42]. The SARP cluster analysis showed that people with the most severe early-onset asthma had greater numbers of skin-test reactions and poorer lung function than individuals with mild asthma and that they were more likely to be of African descent. It also linked severe allergic asthma to a longer duration of disease and a history of pneumonia. These results suggest that both genetic and environmental factors are important in asthma pathogenesis[29]. Interestingly, although IgE is one of the strongest risk factors for severe asthma in people of African descent—in whom age of onset is also significantly earlier than in other racial groups—a similar association of IgE with asthma severity has not been observed in people whose racial background is classified as white[43]. It is likely that as severity of allergic early-onset $T_H2$ asthma increases, non-$T_H2$ immune pathways—including those related to $T_H17$ and $T_H1$—are also engaged (**Fig. 4**), as is innate immunity[44–46].

Clinical phenotyping studies have confirmed the strong family history and probable genetic component to early-onset asthma[25,29]. Genome-wide association studies (GWASs) have shown that there are differences between the genetic factors related to early-onset and later-onset asthma, with stronger genetic links to early age of onset than to atopy and IgE[47,48]. Furthermore, GWASs have indicated that the genes most strongly associated with early-onset asthma, including those encoding IL-33 and its receptor, are epithelial rather than allergy related, although some of them may influence immune function. These results suggest that, despite the relationship between asthma and atopy and allergy, allergy *per se* may not be the pathobiological process that initiates asthma, and that other—perhaps innate—immune and metabolic processes may be important[47] (**Figs. 2** and **4**). In contrast, candidate gene studies carried out by SARP of $T_H2$ pathway–associated genes (including *IL4*, *IL13*, *IL4Rα* and *GATA3*) reported higher numbers of mutations in $T_H2$-related genes with greater severity of disease, supporting the idea that a dose response to $T_H2$-associated gene products is an important factor in high asthma severity[49]. Thus, the relative contribution of allergy- and non-allergy–related genes to both the presence and severity of the early-onset $T_H2$ phenotype remains to be determined.

**Biomarkers and treatment responses.** Most crucial to determining a pathway's importance to a disease is the confirmation that inhibition of that pathway or its activity improves disease outcomes. Corticosteroids are the mainstay of asthma therapy, and many of their beneficial effects result from the modulation of $T_H2$ cytokines and associated inflammation, but their activity is broad and nonspecific[50–52]. Despite this lack of specificity, there is evidence that corticosteroids are most effective in individuals in whom there is evidence of $T_H2$ inflammation as manifested by high FeNO (usually 30–35 parts per billion or higher), sputum eosinophils (≥2% all sputum inflammatory cells) and elevations in airway periostin[35,40,53]. All of these factors have been proposed as biomarkers for $T_H2$ asthma. Notably, these biomarkers are not always present in early-onset asthma of any severity; the exact proportion of people with early-onset asthma who have them remains unknown.

Although most pathobiological studies of asthma have not specifically evaluated early-onset allergic asthma, it is likely that many of the previously studied individuals with mild, corticosteroid-naïve asthma were of this phenotype. Thus, the reported basal increases in $CD4^+$ cells and eosinophils and their reported decrease after treatment with inhaled corticosteroids may represent, at least generally, this phenotype[54,55]. Because inhaled and systemic corticosteroids affect this observed pathobiology in most individuals, it can be difficult to identify a $T_H2$ signature in the airways of corticosteroid-treated people using conventional cellular pathology.

Beyond the nonspecific effects of corticosteroids, treatment with molecules that target components of the $T_H2$ pathway such as antibodies to IgE, IL-4Rα receptor blockers and anti–IL-13 strategies, combined with allergen challenges in individuals with mild, corticosterioid-naïve asthma, has confirmed the relationship of $T_H2$ cytokines to specific allergic asthmatic responses[56–58]. An IgE-specific

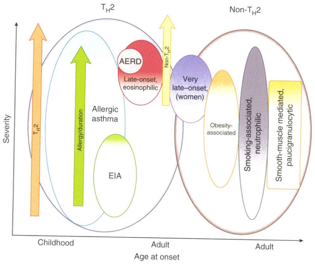
T$_H$2
Non-T$_H$2
AERD
Late-onset, eosinophilic
Non-T$_H$2
Very late–onset, (women)
Allergic asthma
Obesity-associated
Smoking-associated, neutrophilic
Smooth-muscle mediated, paucigranulocytic
Severity
T$_H$2
Allergy/duration
EIA
Childhood
Adult
Adult
Age at onset

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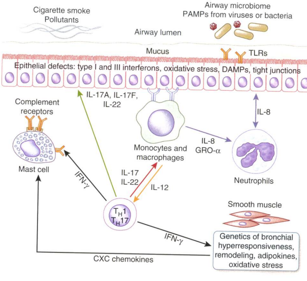
**Figure 3** Theoretical grouping of emerging asthma phenotypes based on the distinction between T$_H$2-high asthma and non-T$_H$2 asthma. T$_H$2 asthma consists of both early- and later-onset disease over a range of severities. It is likely that the majority of early-onset allergic asthma is mild but that an increasing complexity of immune processes leads to greater severity. Later-onset eosinophilic asthma without traditional allergic elements is more likely to be severe, whereas EIA is a milder form of T$_H$2 asthma. Non-T$_H$2 asthma includes very late–onset, obesity-associated asthma as well as smoking-related and neutrophilic asthma, and asthma in which affected individuals show little inflammation. The intensity of the colors represents the range of severity; the relative sizes of the subcircles suggest relative proportions of affected individuals.

antibody (omalizumab) is the only biological agent now approved for asthma. Although IgE-specific antibody treatment has been targeted toward allergic asthma, this classification is loosely defined as minimal elevations of total IgE in the presence of any IgE specific to a particular allergen. With this definition, IgE-specific antibodies affect both early- and late-phase allergic physiological reactions and eosinophilic inflammation[56].

Even more specifically targeting T$_H$2 immunity than antibodies to IgE, the four-week administration of an inhaled IL-4Rα antagonist improved physiological responses to allergen inhalation and decreased FeNO in people with mild, corticosteroid-naïve asthma[32,58]. There may also be a pharmacogenetic response to anti–IL-4Rα treatment, as known risk alleles in the gene encoding IL-4Rα identified participants with better treatment responses[59]. In contrast, a monoclonal antibody to IL-5 did not show efficacy in an allergen-challenge model despite causing profound reductions of blood eosinophils[7]. In addition, two weeks of systemic anti–IL-13 treatment affected physiological responses to allergens but not eosinophilic inflammation[57]. Although the reasons for the differing effects of IL-4 and/or IL-13 from those of IL-5 in allergic responses are not known, the observed efficacy of antibodies to IL-13 in the absence of a reduction in eosinophils and the failure of antibodies to IL-5 despite a reduction in eosinophils suggest that noneosinophilic components may be of greater importance than eosinophils in these specific allergic responses.

Specific T$_H$2 pathway inhibition in nonphenotyped, corticosteroid-treated individuals with chronic asthma has generally been ineffective[5]. In contrast, even modest phenotyping, as shown by the case of antibody to IgE above, improves overall efficacy to some degree, reducing asthma exacerbations and improving symptoms and quality of life[12,13,60]. Yet, as many as 50–60% of individuals did not respond to IgE-specific antibody treatment, particularly those with greater severity of disease, and there are no biomarkers other than IgE to predict response[60,61]. Perhaps the most robust clinical response to IgE-specific antibody therapy was observed in a study of African-American children living primarily in inner-city environments[62], a population enriched for highly T$_H$2-skewed asthma[43,63]. Thus, it remains to be determined whether using a potential T$_H$2 biomarker to define T$_H$2-allergic asthma would improve the likelihood of a response to IgE-specific antibody treatment.

Interestingly, a recent study of treatment with a monoclonal antibody to IL-13, lebrikizumab, showed modest but significant improvements in FEV1 (ref. 64) in people with moderate to severe corticosteroid-treated asthma. Conventional markers of allergic inflammation (IgE, atopy and blood eosinophils) did not define lebrikizumab responders. However, recent studies have suggested that serum periostin, an epithelial protein that is induced by IL-13 and present in greater amounts in the airways of some people with mild asthma, may be a biomarker for a more general T$_H$2 asthmatic phenotype[65–67]. FeNO has also been proposed as a T$_H$2 biomarker because it is produced by inducible nitric oxide synthase, an enzyme that is induced in human airway epithelial cells by IL-13 and present in greater abundance in asthma[33,68,69]. In the lebrikizumab study described above, a subgroup of individuals who had asthma with persistent elevations in serum concentrations of periostin showed greater improvements in airway function and fewer exacerbations after treatment than those with lower concentrations of serum periostin[64]. Interestingly, in a *post hoc* analysis, FeNO levels were as helpful as periostin in identifying T$_H$2-high individuals who would respond to lebrikizumab, but these biomarkers were not compared with the percentages of sputum eosinophils. Although this study suggests that periostin may be an easily obtainable T$_H$2 biomarker, whether it will be better than FeNO or sputum eosinophils remains to be determined in prospective studies.


Cigarette smoke
Pollutants
Airway microbiome
PAMPs from viruses or bacteria
Airway lumen
Mucus
TLRs
Epithelial defects: type I and III interferons, oxidative stress, DAMPs, tight junctions
IL-17A, IL-17F, IL-22
Complement receptors
IL-8
Mast cell
Monocytes and macrophages
IL-8
GRO-α
Neutrophils
IFN-γ
IL-17
IL-22
IL-12
T$_H$1
T$_H$17
Smooth muscle
IFN-γ
Genetics of bronchial hyperresponsiveness, remodeling, adipokines, oxidative stress
CXC chemokines

Debbie Maizels

**Figure 4** Theoretical range of factors that may be involved in the development of non-T$_H$2 asthma. These factors include infection-related elements, T$_H$1 and T$_H$17 immunity, non-T$_H$2–associated smooth-muscle changes including genetics and oxidative stress, and the development of neutrophilic inflammation. IFN-γ, interferon-γ; GRO-α, growth-regulated oncogene-α; PAMP, pathogen-associated molecular pathway; DAMP, danger-associated molecular pathway; TLR, Toll-like receptor.

## Late-onset persistent eosinophilic asthma

**Clinical and biological features.** Eosinophilic asthma is characterized by the presence of eosinophils in higher numbers than normal as determined by sputum, bronchoscopic or blood analysis. Although the definition of eosinophilic asthma is not standardized, studies have indicated that a proportion of sputum eosinophils greater than 2% of all sputum inflammatory cells can distinguish individuals with eosinophilic asthma from healthy individuals and those with non-eosinophilic asthma, suggesting eosinophils as a target for therapeutic intervention[9,11,70,71]. The proportion of asthma associated with high numbers of eosinophils is not known, but studies of mild to severe asthma suggest that it may be around 50%[8,35]. In severe asthma, high numbers of eosinophils can persist despite treatment with inhaled and oral corticosteroids and appear to be consistent over at least 5 years[72].

Persistent sputum eosinophilia (≥2%) despite corticosteroid therapy is associated with an adult-onset, less allergic form of asthma[25,28,29]. This form of asthma is often associated with sinusitis, nasal polyps and sometimes AERD, but there is little to suggest that clinical allergic responses are occurring, despite positive allergen skin tests in ~75% of individuals[25]. A family history of asthma is also less commonly observed than in early-onset disease, and the genetics of this phenotype have not been specifically studied[25,29]. This phenotype is often severe from onset.

The lack of clinical allergy in this phenotype suggests that the $T_H2$ process differs from and is probably more complex than the one associated with the early-onset allergic phenotype. As $T_H2$ cytokines are also upregulated in cancer, inflammatory bowel disease and interstitial fibrosis, a $T_H2$ inflammatory process in the lung without mucosal-specific IgE and associated clinical allergic reactions is clearly possible[73–75]. Some individuals show sputum neutrophilia intermixed with their eosinophilic process[76]. This mixed inflammatory process implies that there are interactions of additional immune pathways with $T_H2$ immunity, including activation of pathways related to IL-33 and IL-17 (refs. 44,77–79).

As noted above, a recognized subphenotype of this late-onset phenotype is AERD, perhaps one of the earliest asthma phenotypes identified. AERD identifies a type of adult-onset, highly eosinophilic asthma with concurrent severe sinusitis, nasal polyps and life-threatening, non-IgE–mediated responses to aspirin and other cyclooxygenase-1 inhibitors[15]. The cysteinyl leukotriene pathway, which is upregulated by $T_H2$ cytokines and present in cells associated with $T_H2$ inflammation (eosinophils, basophils and mast cells)[25,80,81] is upregulated in AERD, and some studies have suggested that the AERD phenotype is linked to leukotriene pathway–related genes[80,82,83]. The degree of overlap between late-onset eosinophilic asthma with AERD and late-onset eosinophilic asthma without AERD is not clear.

**Biomarkers and treatment responses.** Despite the lack of allergy, there is increasing evidence to support a major role for $T_H2$ immunity in this phenotype. Pathologically, IL-13 and IL-5 are present in the lower airways and in association with nasal polyps. Supporting this link, a gene-expression study identified the $T_H2$ biomarker periostin in nasal polyps from AERD[67,84–86]. Other downstream $T_H2$ signature molecules are present in greater amounts in many of these people, including 15-lipoxygenase-1 and its product 15-hydroxyeicosatetraenoic acid, iNOS and eotaxins[67,87,88]. The subset of individuals with AERD and, to some degree, the general late-onset phenotype, is associated with upregulation of the cysteinyl leukotriene pathway, as mentioned above. Finally, the eosinophilia is associated with a thicker SBM and high TGF-β expression, findings that are also observed in early-onset, allergic ($T_H2$-high) asthma[25]. Elevations in FeNO and urinary cysteinyl leukotrienes have also been reported[25,28,89,90]. Whether distinct biomarkers will identify this phenotype remains to be assessed.

Eosinophils are usually highly responsive to corticosteroids, rapidly undergoing apoptosis in their presence[91,92]. It is surprising, therefore, that lung and blood eosinophils persist in a subset of up to 50% of people with corticosteroid-treated asthma, with increasing percentages of these cells seen with increasing disease severity[8]. As early-onset allergic eosinophilic $T_H2$ asthma generally responds well to corticosteroid therapy, this persistent eosinophilia in late-onset disease implies that the $T_H2$ process in this type of asthma is refractory to corticosteroids. The immunobiology underlying the different eosinophil responses in early- and late-onset $T_H2$-related disease is not clear, but high systemic doses of corticosteroids are generally able to overcome this refractoriness in late-onset asthma[93]. As would be expected given the high concentrations of cysteinyl leukotrienes in these individuals, leukotriene modifiers can have a beneficial impact on lung function and symptoms in the AERD subset, even in the face of systemic corticosteroid therapy, further distinguishing the $T_H2$-related processes in early-onset disease from those in late-onset disease[94,95].

The most convincing evidence that $T_H2$ cytokines are present and contribute to the persistent eosinophilia in this phenotype comes from recent drug trials of therapeutics that blocked the IL-4 and IL-13 pathway or the IL-5 pathway[90,96,97]. Results from separate studies of individuals with severe, persistent (and mostly late-onset) eosinophilic asthma treated with a monoclonal antibody to IL-5 showed that this therapy was effective not only in diminishing blood and lung eosinophils but also, importantly, in decreasing exacerbations and systemic corticosteroid requirements[96]. Thus, whether these targeted $T_H2$ approaches will have greater therapeutic efficacy in mild or severe, allergic or nonallergic asthma will be likely to depend more on the amount of remaining $T_H2$ inflammation than on the previously used clinical characteristics.

## Exercise-induced asthma

**Clinical and biological features.** For this discussion, EIA refers to asthma whose symptoms are experienced primarily after exercise. Although this phenotype has been described for years, little is known about its immunological and inflammatory underpinnings. People with EIA often have mild asthma and experience reactive bronchoconstriction (a decline in FEV1 of 10–15%) in response to sustained exercise, and this decline is more frequent and severe in cold, dry conditions. Consistent with a relationship to $T_H2$ processes, this phenotype may be more common in atopic athletes and pathologically associated with high percentages of eosinophils in both sputum and tissue[37,98,99]. However, EIA has also been reported with only low-level eosinophilic inflammation, and the relationship of this form of the disease to $T_H2$ immunity is less clear[99]. EIA has also been associated with mast cells and their mediators, and these cells are consistently associated with $T_H2$ processes[38]. No distinct genetic factors or biomarkers for EIA have been described.

**Treatment responses.** Drugs that modify the cysteinyl leukotrienes also suppress EIA[100,101]. Importantly, monoclonal antibody blockade of the mast cell–promoting cytokine IL-9 (which is linked to $T_H2$ (and $T_H9$) immunity) has also recently been shown to inhibit EIA[102], suggesting that EIA is at least partly $T_H2$ associated. Whether EIA differs from the other forms of $T_H2$ asthma beyond its persistence and severity awaits further study.

### Non-$T_H2$ asthma

Non-$T_H2$ asthma is likely to represent a large proportion of all asthma. However, in comparison to $T_H2$ asthma, little is understood about this asthma subgroup, the phenotypes underlying it or the molecular elements that control it. Non-$T_H2$ asthma may affect 50% or more of corticosteroid-naïve individuals, who, although they meet the criteria for asthma, show less airway obstruction and hyperreactivity than people with $T_H2$-high asthma[35,53]. Many people who have mild to moderate adult-onset asthma and no history of childhood allergic features are likely to fall into this category[103,104]. As these individuals also respond poorly to corticosteroid therapy, in the absence of validated $T_H2$ biomarkers, the proportion of non-$T_H2$ asthma in persistently symptomatic, corticosteroid-treated patients is not clear. However, the lack of efficacy of $T_H2$-targeted therapies in studies of nonphenotyped, corticosteroid-treated patients (even when corticosteroid treatments are tapered) and the existence of multiple other pathways by which airway hyperresponsiveness may develop strongly suggest that a subset of asthma exists with no $T_H2$ immunity[5,105,106].

Apart from the non-$T_H2$ phenotypes discussed below, additional ones are likely to be defined in the future (**Fig. 3**). Individuals who have asthma with little to no inflammation of any type may have a more reactive airway, or their smooth-muscle hyperreactivity may not depend on the immune components known to contribute to hyperreactivity[53,107]. Certain genes related to injury and repair have been targeted for their role in the control of FEV1, and single nucleotide polymorphisms (SNPs) in the genes encoding hedgehog-interacting protein and a disintegrin and metalloprotease 33 have been frequently associated with this parameter in the healthy human population and in people with asthma or other lung diseases[108,109]. The role of $T_H1$ processes is even less understood, although a recent mouse study suggested that interferon-$\gamma$ (IFN-$\gamma$) could enhance mast-cell responses; interestingly, CXC chemokines, induced by IFN-$\gamma$, are known mast-cell chemoattractants to smooth muscle[110,111]. Thus, $T_H2$-independent mast-cell processes could exist in addition to $T_H2$-dependent ones.

### Obesity-related asthma

**Clinical and biological features.** Obesity has been suggested to have a substantial role in the development, control and severity of asthma. Yet, whether obesity is a driving component in asthma development or a mere confounder or comorbidity of its presence remains controversial. Obesity is associated with greater energy expenditure during breathing, deconditioning, shortness of breath and greater likelihood for gastroesophageal reflux and associated coughing and chest tightness, all of which can lead to misdiagnosis of asthma in obese individuals when specific physiological testing is not used[112–115]. Other studies support an association of obesity with a generalized proinflammatory state involving high expression of certain inflammatory mediators such as TNF-$\alpha$, IL-6 and leptins, although obesity is consistently associated with lower amounts of FeNO, fewer eosinophils and, importantly, a diminished response to corticosteroid therapy[116–119]. In fact, weight loss was recently shown to enhance $T_H2$ (and $T_H1$ and $T_H17$) cytokine production from peripheral blood lymphocytes[120].

Interestingly, a distinct obesity-related asthma phenotype seems to occur only in non-$T_H2$ asthma[120,121]. In early-onset allergic $T_H2$ asthma, the disease duration correlates with body mass index, and a higher body mass index is likely to be related to lower activity levels and more use of corticosteroids; however, no such relationship to duration exists in late-onset, less-allergic asthma that is obesity related[120,121]. Two unbiased studies also supported the existence of a later-onset, non-$T_H2$ obesity-related phenotype by identifying a group of women, most of whom were obese, who had late adult-onset (mid-40s), minimally allergic asthma with a high burden of symptoms but little of the need for high-intensity healthcare (such as time in an intensive care unit or need for intubations) typically seen in early-onset allergic asthma[28,29]. Despite similarities in the cluster approaches used, each study reported different levels of eosinophilic inflammation, suggesting complexities beyond obesity-related hormonal interactions alone.

**Biomarkers and treatment responses.** Although adipokines have been proposed, no specific biomarkers have been accepted to define obesity-related asthma, perhaps because the role of obesity differs between $T_H2$ and non-$T_H2$ asthma. Although weight-loss programs and bariatric surgery have been reported, anecdotally, to improve the symptoms of asthma (and even cure it), the relationship of obesity to objective measures of inflammation and physiology is unclear[122]. In the most convincing study to date, profound weight loss achieved after bariatric surgery in a group of people who had nonallergic, late-onset (non-$T_H2$) asthma was associated with improvements in symptoms, quality of life and bronchial hyperresponsiveness[120]. In contrast, similar weight loss in obese individuals with allergic ($T_H2$) asthma did not improve bronchial hyperresponsiveness, and the high $T_H2$ cytokine production in these individuals suggests that weight loss may even worsen $T_H2$ asthma. Thus, weight loss as a therapy for obese-associated asthma seems to be more beneficial when the asthma is not associated with $T_H2$ inflammation. The poor clinical responses to corticosteroids that have been observed in obesity-related asthma may also be due to the lack of association of this phenotype with $T_H2$ inflammation, which is traditionally a corticosteroid-responsive process[53,117,119]. Whether this lack of corticosteroid responsiveness supports studies that have suggested that more corticosteroid-refractory pathways—including those involving IL-6 or TNF-$\alpha$, either alone or in association with increased oxidative stress—are important in driving disease awaits further study[123].

### Neutrophilic asthma

**Clinical and biological features.** Neutrophilia has been inconsistently associated with asthma and severe asthma for several years[24,124]. Data to support neutrophilic asthma as a specific phenotype remain modest, and no consensus exists as to what level of neutrophilia should define the phenotype. Neutrophilia is generally seen in corticosteroid-treated patients. Corticosteroids inhibit neutrophil apoptosis and, in some settings, contribute to neutrophil activation, suggesting that corticosteroid treatment itself is likely to have some role in the development of neutrophilia[125,126]. In affected individuals, lung neutrophilia has been associated with lower lung function, more trapping of air, thicker airway walls (as measured by computed tomography scans) and greater expression of matrix metalloproteinases than are seen in people with non-neutrophilic asthma, but it has not been associated with airway hyperresponsiveness[127–131]. Although lung neutrophils have not been incorporated into any unbiased analysis, in a *post hoc* analysis, sputum neutrophilia was greatest in a SARP cluster of individuals who had generally adult-onset and severely obstructed (and incompletely reversible) asthma and the highest-intensity healthcare usage and systemic corticosteroid use[29]. The relationship of this phenotype to smoking remains unclear and inconsistent. Although the SARP cluster study did not include smokers, some studies suggest that when the data are controlled for corticosteroid use and smoking, this phenotype does not exist[132,133]. An additional sputum gene-expression profiling study in Australia reported that neutrophilic inflammation was associated with upregulation in IL-1 and TNF-α pathways, but there was little association with any clinical phenotype[133]. Neutrophilia can also co-exist with eosinophilia, and this identifies the people with the most severe asthma and emphasizes the complexity of the immunobiology of severe asthma in which multiple different innate and adaptive immune pathways and cells may have roles[76,134].

**Treatment responses.** Corticosteroids are less effective in this phenotype, perhaps because of the absence (or suppression) of a $\mathrm{T_H}$2 process[10]. A single study suggested that neutrophilic asthma may be more responsive to treatment with macrolide antibiotics, with a reduction in the expression of neutrophilic markers and improvement in quality of life, but no improvement in asthma control or FEV1 in treated individuals[135]. Whether the observed improvement was caused by the antibiotic or anti-inflammatory effects of macrolides is not clear. The lack of neutrophil-targeted interventions limits the ability to determine whether neutrophilia is a biomarker or a target for therapy. However, the lack of efficacy of anti–TNF-α in severe asthma treated with high doses of corticosteroids raises questions about the importance of TNF-α in neutrophilic asthma[136].

$\mathrm{T_H}$17 inflammation has been strongly linked with neutrophilia and envisioned as a therapeutic target, and this has led to the development of mouse models that overexpress IL-17 and are neutrophilic and corticosteroid resistant[34,44,45]. Expression of both IL-17A and IL-17F has been reported to be high in severe asthma in association with poor lung function, but, interestingly, these cytokines have not been linked to neutrophilic inflammation or clinical parameters[78,137]. Thus, the efficacy of IL-17–targeted therapy in human neutrophilic (or other) asthma phenotypes awaits results from ongoing clinical trials.

## Conclusions and future directions

The concept of asthma phenotypes is rapidly evolving from one that was focused on clinical characteristics to one that links underlying biology to phenotype. There is strong evidence supporting a $\mathrm{T_H}$2-high phenotype in up to 50% of people with asthma of any severity, yet 50% show no evidence for this immune process. Even the percentage of early-onset allergic asthma that is truly $\mathrm{T_H}$2 high remains unknown. As the complexity and severity of the disease presentation increases, the accompanying immunopathology becomes more complex and is likely to include additional adaptive immune elements and structural changes in the airways. Asthma of any severity that presents without evidence for $\mathrm{T_H}$2 immunity remains poorly understood. Although there has been substantial interest in the role of $\mathrm{T_H}$17 immunity in neutrophilic (and even obesity-related) asthma, support for this theory in humans is modest. Thus, our understanding of phenotypes remains incomplete.

The future understanding of these phenotypes will require integrating bench and bedside approaches. Data from ongoing genomic and proteomic profiling studies of large, well-characterized asthma populations should allow the development of improved animal models of asthma that mirror more closely the inflammatory (or noninflammatory) environment identified from the human studies. To better represent the non-$\mathrm{T_H}$2 phenotype, studies in animal models will need to focus on infectious and other environmental elements, obesity and hormonal changes in the absence of allergic sensitization and challenge. Genetic studies of mice that are particularly hyperreactive in the absence of inflammation could also provide clues into the biology underlying this phenotype, perhaps for milder asthma in particular. In humans, population studies of a range of asthma severities will require longitudinal components to characterize molecular phenotypes and their stability over time, validate genomics at the protein or lipid level and develop new technologies that enhance the quality of the results that can be obtained from very small samples.

Although our understanding of even mild to moderate asthma remains incomplete, severe and poorly controlled asthma phenotypes remain the great unmet clinical need. To further our understanding of severe asthma, new molecular targets that are first identified using human 'omics' studies will need to be mechanistically evaluated in animal and cell-culture models to better define their functionality, association with accompanying pathways and relationship to specific phenotypes. However, the ultimate test of a phenotype is the efficacy of a targeted molecular approach. It is hoped that these combined bench and bedside approaches will enhance the successful development of personalized and phenotype-specific therapies for asthma.

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# Viral infections and atopy in asthma pathogenesis: new rationales for asthma prevention and treatment

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**Prospective birth cohort studies tracking asthma initiation and consolidation in community cohorts have identified viral infections occurring against a background of allergic sensitization to aeroallergens as a uniquely potent risk factor for the expression of acute severe asthma-like symptoms and for the ensuing development of asthma that can persist through childhood and into adulthood. A combination of recent experimental and human studies have suggested that underlying this bipartite process are a series of interactions between antiviral and atopic inflammatory pathways that are mediated by local activation of myeloid cell populations in the airway mucosa and the parallel programming and recruitment of their replacements from bone marrow. Targeting key components of these pathways at the appropriate stages of asthma provides new opportunities for the treatment of established asthma but, more crucially, for primary and secondary prevention of asthma during childhood.**

Asthma is a common disease affecting millions of children and adults. In its chronic form, asthma has a complex pathology that is characterized by airway inflammation accompanied by alterations in patterns of vascularization, innervation and airway smooth muscle growth and disturbances of the epithelial-mesenchymal trophic unit throughout the conducting airways[1]. These changes collectively compromise an individual's capacity to maintain normal respiratory functions, particularly when exposed to ubiquitous airborne environmental stimuli. This pathological state stems from repeated episodes of airway inflammation and ensuing cycles of tissue repair and regeneration occurring over a period of years (**Fig. 1**, top). This results in cumulative structure and function changes in the lung that eventually exceed critical thresholds beyond which the 'persistent asthma phenotype' comes to represent the new norm (**Fig. 1**, bottom).

In terms of asthma therapeutics, although effective symptom-relieving treatments are available, attempts to develop disease-modifying drugs have met with limited success. This slow pace of drug development is a reflection of the complexity of the underlying pathogenic mechanisms of asthma. However, this complexity is not universally acknowledged, and in the quest for new drugs, the strong tendency is still to view the disease process in unidimensional terms and focus exclusively on established asthma. This view is exemplified by the approach to the most common form of this disease, atopic asthma, in which the identity of the perceived primary pathogenic mechanism is enshrined in the name of the disease itself. Superficially, it seems that it could not be more straightforward: atopic sensitization leads to airway inflammation, which results in asthma. This sequence of events is readily demonstrable in experimental models, findings from which have driven the development of a range of therapeutic agents, such as a monoclonal antibody (mAb) to interleukin-5 (IL-5), that are effective in blocking their specific allergy-associated targets in short-term trials in subjects with established atopic asthma but do not achieve long-term disease modification. We do not share the view held by some in this field that the underlying experimental approach to understanding asthma pathogenesis that led to the development of these drugs is flawed; however, we believe that much of the knowledge gained from the experimental approach continues to be applied clinically in suboptimal contexts. In this regard, we share the growing view that whereas aeroallergen sensitization is one of the strongest asthma risk factors, it rarely *per se* leads directly to persistent asthma. Instead, this sensitization most frequently acts in synergy with other proinflammatory environmental cofactors, most notably respiratory viral infections, to drive disease development. The nature of the interactions between antiviral and atopic pathways and how they may be potentially targeted to treat and/or prevent asthma is the focus of this review.

## Mechanisms driving asthma initiation: birth cohort studies

The potential role of respiratory viral infections as an initiating factor in asthma pathogenesis has been recognized for many years[2], but a more comprehensive understanding of the magnitude of the impact of these infections has only become evident relatively recently in light of emerging results from long-term birth cohort studies in the United States[3,4], Australia[5,6] and Europe[7] that span decades (reviewed in ref. 8).

These studies have collectively showed that whereas wheeze is common during infancy—particularly in association with respiratory infection—expression of this phenotype is usually transient and resolves spontaneously by age ~3 years (**Box 1**). In a subset of children, however, wheeze consolidates into a persistent clinical pattern that is indicative of early

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onset asthma, and this phenotype is strongly associated with early sensitization to aeroallergens. In addition, lower respiratory viral infections (LRIs) can predispose to the development of early asthma, conferring a slightly higher risk than atopy alone. However, the most severe childhood asthma, and the type that confers the highest ensuing risk for progression to persistent asthma, is encountered when LRIs occur against a background of pre-existing aeroallergen sensitization[5,6,8–10], in particular during the period when postnatal lung growth and differentiation are proceeding most rapidly (**Box 1**). Under these circumstances, affected children are tenfold to 30-fold more likely to develop persistent and/or severe disease[6,9–11], and this outcome is a reflection of both the LRI frequency and intensity and the amounts of aeroallergen-specific IgEs that are present at the time of these episodes[5].

A key underlying issue is the relative strength of the associations between different respiratory viruses and early asthma pathogenesis. Earlier studies emphasized the key role of respiratory syncytial virus (RSV)[12], however, recent attention has shifted toward rhinovirus[3,10,13], especially the frequently encountered rhinovirus type C[14]. However, RSV is a major pathogen in infants[15], and the relative roles of these viruses in driving asthma beyond childhood are not completely understood[16].

### Progression of asthma beyond early childhood

The question of what sustains asthma progression beyond the preschool years is central to the development of improved treatments, and cohort studies have again been instructive in this area. The strength of the association between atopy and asthma in schoolchildren was first recognized in the 1980s, but the clinical relevance of this association was not initially widely appreciated. These findings have proven to be extremely robust over time, as exemplified by recent studies in teenagers, particularly the clear quantitative associations between the strength of T helper type 2 ($T_H2$) cell immunity against indoor aeroallergens and asthma severity[17]. This connection, however, is not absolute—whereas the majority of teenagers with asthma are atopic, only a minority of these individuals with atopy develop asthma[17]. This suggests that although atopy manifesting as an inhalant allergy constitutes a strong risk factor for asthma in this age group, it is usually insufficient to trigger full-blown disease on its own, and additional cofactor(s) must be involved.

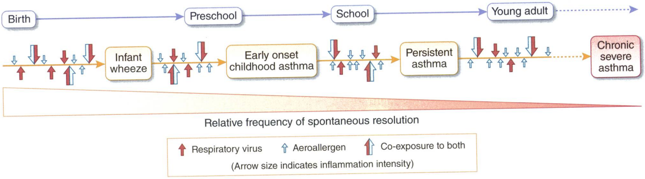
Epidemiological clues as to the nature of these cofactor(s) have come from studies on environmental triggers that precipitate wheezing attacks. Controlled exposure of aeroallergen-sensitized individuals with asthma to aerosolized allergens can provoke wheeze accompanied by $T_H2$ cell–associated airway inflammation[18], mirroring the murine asthma models. However, the extent to which direct aeroallergen-induced triggering explains spontaneous asthma attacks is unknown, as a clear clinical history of acute asthma after allergen exposure is rarely obtained. At the severe end of the asthma exacerbation spectrum, as exemplified by episodes that are associated with school absenteeism or hospital admission (reviewed in ref. 8), the most notable clinical feature is the concomitant presence of viral LRI. A variety of evidence suggests that this susceptibility is determined in part by aberrations in host antiviral immunity involving type 1 and/or type 3 interferons (IFNs)[19–22]. However, it is noteworthy that >80% of affected children are also atopic, and this comorbidity is a prominent feature of severe asthma exacerbations among adults as well[8]. This pattern is also reflected in children with less severe asthma, among whom the severity and duration of respiratory-virus–associated illnesses and the accompanying loss of asthma control are associated with an underlying atopy[11]. Emerging evidence suggests that additional features of these more severe asthma episodes are covert interactions between virally-triggered and allergen-triggered inflammatory pathways, as discussed below.

### Viral-induced perturbation of immunological homeostasis

As noted above, viral infections perturb a range of mechanisms that are central to the maintenance of immunological homeostasis in the airways.

## BOX 1 Initiation, progression and persistence of asthma in individuals with atopy

Normal lung function develops along percentiles[76,77] in a fashion similar to normal height, and disturbance to lung growth in early life can alter lung function permanently. The relevant lung growth processes, which proceed most rapidly during the first 2–3 years of life, include (i) gross structural alterations, such as progressively increasing airway diameter; (ii) more subtle processes, including alveolarization and associated changes in the airway-lining epithelia; and (iii) establishment of neural control of airway smooth muscle and local irritant receptor systems[78]. It has become apparent that environmental exposures that interfere with normal lung growth during this crucial period have the potential to limit an individual's capacity to attain optimal respiratory function[79,80]. The result of this interference is that the lung growth trajectory of an affected individual is dislodged, potentially permanently, onto a lower percentile line, and, thus, the individual is at an increased risk for asthma. A classic example is maternal tobacco smoke exposure during pregnancy that results in reduced respiratory function in her offspring at birth, which subsequently 'tracks' into later childhood and adulthood. Additionally, postnatal inflammatory insults to the growing lung that are associated with viral infection[4] and acute asthma exacerbations[81] also exert potent deleterious effects on the development of healthy respiratory function that can persist into later life. The precise mechanisms underlying this tracking phenomenon are probably multifactorial in nature and may include tissue remodeling changes associated with alterations in airway epithelial and myelofibroblast populations (the epithelial-mesenchymal trophic unit[82,83]).



A key cell population in this regard comprises the airway mucosal dendritic cells (AMDCs), which express the immature antigen-surveillance phenotype that they normally maintain until migration to the regional lymph nodes (RLNs), where they deliver a signal that is weakly $T_H$2 polarized that precedes the induction of tolerance[23]. During the course of viral infection, murine respiratory tract dendritic cell populations go through a variety of changes that potentially influence the tonus of the local immune surveillance mechanisms. One such change is the accumulation of plasmacytoid dendritic cells (pDCs)[24], which normally comprise a minor subpopulation in resting airway mucosa. These cells are postulated to play a crucial part in mucosal tolerance[25] and are also a major source of the IFNs that mediate the primary defense against viral infection.

A feature of ongoing immune surveillance within the airway mucosa is the orderly turnover of resident myeloid dendritic cells (mDCs), as replacements for emigrating antigen-bearing mDCs trafficking to the RLNs are continuously recruited from bone marrow[23]. In rats, this process is markedly accelerated during parainfluenza infection[26]. Comparable AMDC mobilization has been confirmed in mouse models[24,27] and in virally infected children[28]. Notably, expansion of the AMDC population persists well beyond parainfluenza clearance[26], and similar findings apply to RSV infection[24,27,28]. The mechanistic basis for these persistent post-viral effects on AMDCs remains to be determined.

It is noteworthy that marked changes have been observed in other lung cell populations in infected mice that also persist after viral clearance[29]. Prominent among these populations are macrophages expressing the 'alternative activation' phenotype that is dependent on IL-4 or IL-13, and similar cells have been reported in lung biopsies from a small sample of patients with asthma and chronic obstructive pulmonary disease[29] and from bronchoalveolar lavage fluid derived from subjects with pulmonary fibrosis[30]. More detailed study of the activation phenotype of lung macrophages in individuals with asthma after exacerbation merits high priority given the long life span of these cells and, thus, their potential to influence the local tissue milieu for prolonged periods after a transient activation event.

## Linking antiviral and atopic inflammatory pathways

Recent investigations have suggested that in addition to changes in the population dynamics of lung myeloid cell populations, viral infections may also profoundly influence their functional phenotype. Of particular relevance to this discussion are findings from Grayson and colleagues showing type 1 IFN–dependent induction of the high-affinity IgE receptor (FcεR1) on lung dendritic cells in the wake of murine parainfluenza infection[31,32]. The authors suggested that subsequent crosslinking of this receptor by viral-specific IgE may lead to the chemokine (C-C motif) ligand 28 (CCL28)-mediated recruitment and subsequent local activation of virus-specific $T_H$2 memory cells, thus providing a secondary source of proinflammatory cytokines in the already inflamed airways. The potential implications of a similar mechanism operating against a background of conventional aeroallergen sensitization were not explored in this model. However, a more recent study on the effects of influenza in mice sensitized to house dust mites confirmed upregulation of FcεR1 in infected lung tissues and linked this upregulation with an increased responsiveness to concomitant exposure to aerosolized house dust mites, which manifested as enhanced $T_H$2 cell–associated airway inflammation and decreased lung function[33].

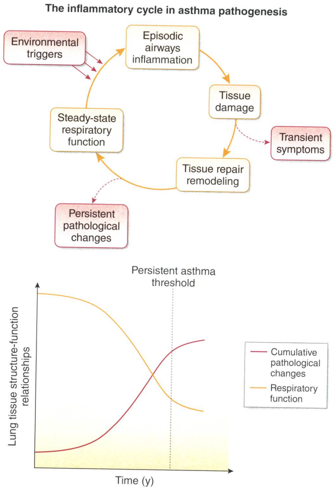

The inflammatory cycle in asthma pathogenesis
Environmental triggers
Episodic airways inflammation
Tissue damage
Transient symptoms
Tissue repair remodeling
Steady-state respiratory function
Persistent pathological changes
Persistent asthma threshold
Lung tissue structure-function relationships
Time (y)
Cumulative pathological changes
Respiratory function

**Figure 1** The inflammatory cycle in asthma pathogenesis. Asthma development is driven by repeated cycles of inflammation triggered by airborne irritant stimuli (top). Symptoms are initially intermittent and are associated with acute inflammation and edema and intermittent airway narrowing. Over time, the resolution of inflammation between clinically apparent episodes of asthma becomes less complete. Persistent inflammation leads to repeated cycles of tissue repair and regeneration, which may themselves be aberrant, and can lead to pathological changes that persist for long periods. As these changes accumulate, they lead to progressive deterioration in respiratory function (bottom). Once these changes exceed a critical threshold, they may not be reversible and may result in persistent asthma, with persistent symptoms that are not easily controlled by currently approved medications.

## Severe asthma exacerbations in children: recent insights

As has been previously noted[8], a small subset of asthma exacerbations in children are sufficiently severe to require hospitalization, and the children with these severe exacerbations typically have ongoing high-level inhalant allergy together with a concurrent respiratory viral infection. We recently studied a cohort of such subjects[34] using an approach that was derived from earlier literature, which established that challenge sites in the lung signal to the bone marrow to recruit replacements for the myeloid cells that are engaged in antigen clearance during inflammatory episodes[35]. This process also involves selective preprogramming of effector functions that are dictated by the mix of incoming inflammatory signals reaching the bone marrow, thus equipping emigrating cells to deal optimally with the specific challenge involved[36]. Sampling blood-borne cell populations during inflammation as opposed to at baseline may provide some insight into the nature of the signals emanating from the challenge site and the resultant functional programming of migratory effector cells without directly accessing the challenge site itself.

Adopting this approach, we profiled circulating peripheral blood mononuclear cells from children with atopy who were hospitalized for severe viral-induced asthma using a combination of microarray, quantitative RT-PCR and flow cytometry, comparing samples collected at admission to those collected during the subsequent convalescence[34]. The most prominent exacerbation-associated signatures in these subjects were within circulating myeloid populations (monocytes, mDCs and pDCs) and were indicative of IL-4– or IL-13–dependent activation and type 1 IFN signaling. Moreover, strong quantitative relationships were observed between the expression of individual genes and the intensity of the exacerbation[34]. These included *CCR2*, encoding chemokine (C-C motif) receptor 2, a major chemokine receptor directing migration to inflamed lung[37], together with *IL13RA2* (encoding IL-13R) and *CD1d*; upregulation of these genes was reproduced *in vitro* by the incubation of monocytes with recombinant IL-4 or IL-13 (ref. 34). The myeloid exacerbation signature also included FcεR1-γ, which was inducible *in vitro* by type 1 IFN, and parallel upregulation of FcεR1-α was shown both on monocytes and on the major dendritic cell subsets using flow cytometry[34].

The cytokine signals that are relevant to this systemic mechanism are probably transmitted from the airways to the bone marrow in a cell-associated form (**Fig. 2**). Indeed, the bone marrow is now recognized as a key repository for migratory T memory cells[38], and the *in situ* activation of these cells by immigrant antigen-bearing dendritic cells has also been formally shown[39]. Additionally, preactivation of these T memory cells may occur at sites of allergic inflammation, as has been inferred in human studies that identified IL-5–secreting $T_H2$ cells in bone marrow aspirates immediately after bronchial allergen challenge[18]. Type 1 IFN–dependent preprogramming of lung-homing leukocytes in bone marrow has also been shown during respiratory viral infection[40], and although the source of the IFN signal was not identified, the pathway through which tissue-derived dendritic cells can bypass the RLNs and traffic directly to bone marrow[39] may be involved.

It is noteworthy that FcεR1-α is known to be constitutively hyperexpressed on myeloid cells in individuals with atopy[41], and the translocation to the cell surface and stabilization of FcεR1-α as functional FcεR1-αγγ complexes is limited by the availability of the γ chain[42,43]. Consequently, upregulation of the γ chain gene in myeloid cells from individuals with atopy by type 1 IFN, as was shown in our *in vitro* studies[34], provides a potential mechanism for increasing the overall expression of functional FcεR1, similar to what has been reported in the atopic asthma mouse model[32,33] (**Fig. 2**). If this is the case, viral-triggered release of type 1 IFN in the infected airway may initiate this cascade through direct effects on resident AMDCs, and this cascade may be further amplified as inflammation associated with $T_H2$ cells develops in the mucosa and the resultant cell trafficking delivers the optimal combination of cytokine signals required to drive expression of both the FcεR1-α and γ chain genes in the myeloid precursor compartment. This mechanism may explain the presence in the circulation of monocytes and dendritic cells bearing functional FcεR1 in conjunction with gene signatures that are indicative of biomarkers of the alternative activation phenotype, which includes the FcεR1 γ chain itself plus the chemokine receptors required to facilitate their homing to the lung and airways to replenish local populations[34]. This systemic mechanism may function in virally infected children with atopy in parallel with the locally operating mechanisms described in murine parainfluenza models that stimulate the expression of the same range of $T_H2$-associated phenotypic markers on resident lung and airway myeloid cells[29,31,32].

One consequence of FcεR1-αγγ upregulation on AMDCs in virally infected individuals with atopy is suggested by observations relating to the pathogenesis of atopic dermatitis. Notably, studies on lesional sites in atopic dermatitis have indicated that if local dendritic cells armed with functional FcεR1 are coexposed to specific IgE and allergen, their capacity to capture, process and present activation signals to $T_H2$ memory cells is markedly amplified[44,45]. The preconditions of local availability of specific IgE and the relevant allergen would probably be met in the airways of many exacerbation-prone children with asthma among whom presensitization to perennial aeroallergens is common[8]. If both the specific IgE and relevant allergen are present, increased FcεR1-αγγ expression on the AMDCs triggered by virus should result in enhanced $T_H2$ cytokine release in the airway mucosa, increasing local inflammation in the infected airway (**Fig. 2**). A further consequence of increased FcεR1-αγγ expression may be a prolongation of the initiating infection itself through $T_H2$ cell–mediated antagonism of antiviral immunity, mirroring 'immune evasion' strategies that are used by a range of other pathogens[46,47]. This conclusion is consistent with a recent report on sputum-derived cells collected from children during severe viral-induced asthma exacerbations indicating the upregulation of $T_H2$-associated gene signatures, including *Fcer1a, IL5* and *IL13* with reciprocal downregulation of the $T_H1$-like/cytotoxic effector pathway[48]. It is also pertinent to mention a recent report that found that triggering of FcεR1 on pDCs attenuates their capacity to produce type 1 IFNs[49], thereby blunting the primary antiviral defense.

Protection against the spread of pathogens beyond the initial infection site is the primary role of type 1 IFNs, and the LRIs that are associated with asthma exacerbations reflect an initial failure of these primary defenses. Airway epithelial cells from individuals with asthma reportedly have a reduced capacity for the production of type 1 and type 3 IFNs[20,21], which may increase these individuals' susceptibility to infection. However, in terms of asthma exacerbations, the implications of variations in IFN-response capacity may be more complex and context dependent, given that several IFN-producing cell types (epithelia, neutrophils and pDCs) may be involved at various stages in the exacerbation cycle. Additionally, we have shown that although IFN-α efficiently activates myeloid FcεR1-γ gene transcription if present at sufficiently high concentrations, it can also provide feedback inhibition of IL-4– or IL-13–mediated FcεR1 α-chain induction[34], which is consistent with a longstanding reports in the literature regarding the allergy-antagonistic effects of this cytokine. This suggests that the same type 1 IFN signal that potentially initiates this FcεR1-dependent cascade through direct effects on resident AMDCs may eventually terminate the bone-marrow–dependent amplification loop that sustains the response, provided it accumulates in sufficient quantities in the bone marrow.

## FceR1 expression on myeloid precursors during the 'atopic march'

Whereas the overall scenario described above (**Fig. 2**) may be restricted to the more severe manifestations of virus-associated asthma, the underlying mechanism involving communication between atopic lesional sites and myeloid precursor populations seems to operate covertly at lower intensities across a broad atopic disease spectrum. Three observations are consistent with this suggestion. First, blood monocytes from individuals with atopy constitutively hyperexpress FcεR1-α at levels that correlate strongly with their serum IgE titers[41], which can now be understood as precursor activation through a chronic drip feed of $T_H2$ cytokine signals from sites of allergic inflammation to the bone marrow. Second, IL-5 signals triggered in the airways by aeroallergen challenge of individuals with atopic asthma[18] (and sensitized mice[50]) leads to the activation of eosinophils in bone marrow and their subsequent trafficking back to the lung. And third, findings have indicated that during active flares of atopic dermatitis, rhinitis and atopic asthma, FcεR1-α expression is upregulated on dendritic cells in the respective target tissues (relative to when disease is quiescent) and that this transient upregulation is mirrored in dendritic cell populations in unaffected tissues in the same subjects[51]; this 'spillover' phenomenon is explicable via the operation of the atopic lesion–bone marrow axis, given that dendritic cell populations in all tissues are continuously renewed from the same precursor compartment.

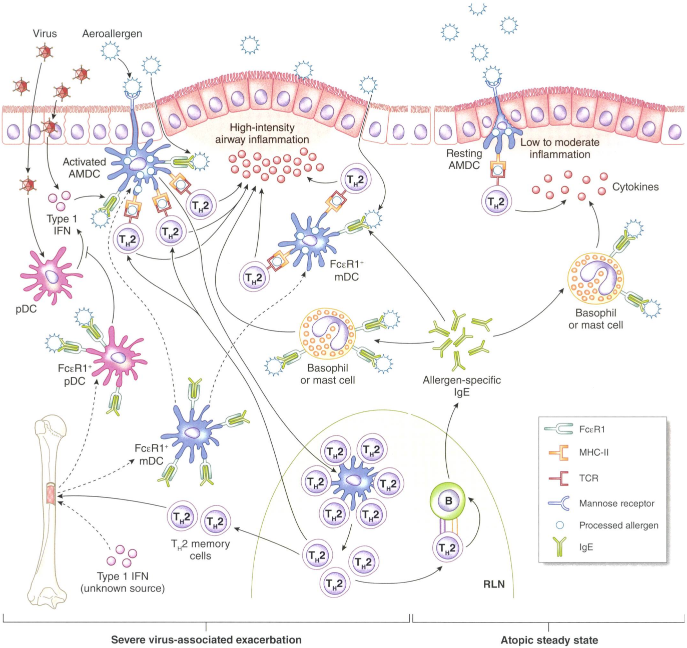

Virus
Aeroallergen
High-intensity airway inflammation
Activated AMDC
Type 1 IFN
$T_H2$
pDC
FcεR1⁺ pDC
FcεR1⁺ mDC
Basophil or mast cell
Allergen-specific IgE
Resting AMDC
Low to moderate inflammation
Cytokines
$T_H2$ memory cells
Type 1 IFN (unknown source)
B
RLN
FcεR1
MHC-II
TCR
Mannose receptor
Processed allergen
IgE
Severe virus-associated exacerbation
Atopic steady state

**Figure 2** Cellular processes underlying virus-associated atopic asthma exacerbations. In individuals with stable atopic asthma, ongoing aeroallergen exposure sustains a state of relatively low level T cell–mediated and granulocyte-mediated inflammation in the airway epithelium that is punctuated occasionally by episodes of moderate intensity inflammation, for example, when aeroallergen exposure levels 'spike'; however, the potential for the local expression of high-intensity $T_H2$ cell–mediated inflammation is normally limited by restriction of the functional phenotype of the resident AMDCs to antigen uptake and processing with minimal presentation. This ongoing 'steady state' is perturbed by the local release of type 1 IFN in response to viral infection, which sets in motion a cascade that is initiated by the upregulation of turnover and FcεR1 expression on resident AMDCs, leading to increased recruitment and local (mucosal) activation of $T_H2$ effectors from the RLN and the subsequent expansion of $T_H2$ memory cell clones in the RLN by migrating AMDCs. The ensuing translocation of cytokine signals to the bone marrow by migrating $T_H2$ memory cells together with a source of type 1 IFN results in the release into the circulation of functionally upregulated myeloid populations, which home to the lung to further amplify local inflammatory responses. An additional short-term consequence is attenuation of local type 1 IFN production by pDCs through activation of their surface FcεR1, potentially contributing to viral persistence. The kinetics of this overall process are incompletely understood and may be highly variable; in particular, upregulation of myeloid cell populations in the lung and airways that persists long after viral clearance has been reported in both human and experimental systems, and the underlying mechanisms for persistence are unknown. B, b cell; MHC-II, major histocompatibility complex type 2.

These observations, together with those discussed in the previous section, suggest a plausible mechanism for what has been termed the 'atopic march', which is the characteristic pattern of atopic disease development during childhood involving the progression from initial atopic dermatitis and/or food allergy to rhinitis and eventually asthma[52] (**Fig. 3**). The operation of this axis provides a means by which active atopic disease in one organ system can facilitate the expression of disease in a secondary (previously unaffected) tissue by enhancing the efficiency of the functions of local antigen-presenting cells through myeloid FcεR1 upregulation, thus optimizing the conditions for the local activation of allergen-specific $T_H2$ memory cells in the secondary tissue. The example in **Figure 3** draws on the literature showing, first, that aeroallergen sensitization in the absence of asthma symptoms is the rule rather than the exception[17] and, second, that allergic rhinitis is a strong risk factor for subsequent asthma[52]. In the scheme shown, active expression of allergic inflammation in the nose (rhinitis) increases the likelihood that inhalation of an aeroallergen will trigger clinically relevant levels of $T_H2$ cytokine secretion in the conducting airway mucosa by indirect upregulation of local FcεR1-dependent functions of the antigen-presenting cells. Over time, this may accelerate the progression toward expression of persistent airway disease (asthma), which is dependent on accumulation of pathological changes above a critical threshold in target tissues (**Fig. 1**, bottom).

It is worth noting that a related mechanism has been proposed based on murine experimental atopic dermatitis studies showing aggravation of asthma-like manifestations in the airways through hyperproduction of thymic stromal lymphopoietin (TSLP) by epidermal keratinocytes[53,54]; recent evidence suggests that this aggravation may also be mediated by effects in the bone marrow, possibly involving the stimulation of basophil hematopoesis[55].

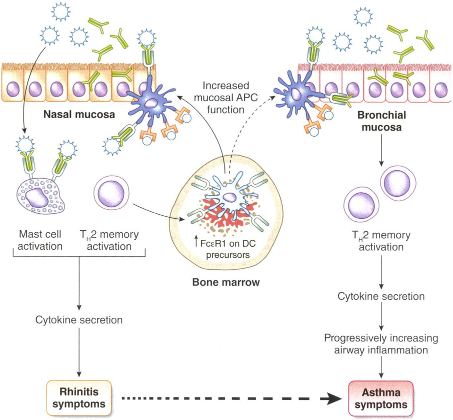


**Figure 3** The atopic march unmasked? Findings summarized in the text provide a plausible mechanism for a longstanding but poorly understood clinical paradigm in the allergy field, notably the atopic march, which is exemplified by progression from allergic rhinitis to asthma. DC, dendritic cell.

## The respiratory microbiome: a role for bacteria in asthma?

Opportunistic bacterial infections have long been recognized as potential contributors to the morbidity associated with viral LRIs, and this issue has arisen again in relation to the link between respiratory infections and asthma in light of two lines of recent evidence. First, using traditional culture methodology, it has been shown that early nasopharyngeal colonization with bacterial pathogens is associated with an increased risk for childhood asthma[56]. Recently, the application of sensitive metagenomic technology has revealed the presence in airway aspirates and tissue samples of a hitherto unrecognized microbiome comprising multiple bacterial strains[57,58]. These findings relate both to healthy individuals and individuals with asthma. Although bacterial diversity and density are increased in individuals with asthma, these are nevertheless multiple species that are also present in healthy individuals, suggesting that efficient homeostatic mechanisms must operate continuously in the lower respiratory tract to maintain a local steady state. Nevertheless, the constant presence of bacteria on the airway surface, particularly in atopic asthma, in which disturbance of epithelial integrity secondary to local inflammation is a characteristic feature, poses a series of quandaries relating to pathogenesis which have yet to be addressed.

From an immunological perspective, one window into the underlying relationships between a host and its respiratory microbiome is through an assessment of humoral immunity to index organisms, and some recent studies on the responses to protein antigens from *Haemophilus* and *Streptococcus* have provided interesting pointers for the future. Children who develop early asthma and atopy have lower serum concentrations of bacterial-specific IgG1 relative to the overall population[59,60], as do children who are susceptible to viral-triggered asthma exacerbations[61]. Reduced IgG1 concentrations in this context suggest an increased susceptibility to transmucosal bacterial incursion and may also connote underlying deficiencies in immune surveillance mechanisms. In this context, it is pertinent to note reports suggesting enhanced susceptibility to invasive pneumococcal disease in individuals with asthma[62,63].

Providing a second line of evidence, IgE responses against *Haemophilus* and *Streptococcus* are found in virtually all teenagers and, surprisingly, associate with a reduced susceptibility to asthma[64]; follow-up studies in 5-year-old subjects indicated a similar relationship[60]. Based on an extensive literature from other areas of inflammation biology, it seems probable that this bacterial-specific IgE may be a surrogate for underlying $T_H2$ cell immunity, especially for specific $T_H2$ memory cells producing IL-4 or IL-13. In this context, one of the most potent inflammatory responses involves interactions between bacteria and tissue macrophages, triggering the secretion of cytokines including IL-1 and tumor necrosis factor $\alpha$ (TNF-$\alpha$), and this process is strongly inhibited by IL-4 or IL-13 (reviewed in ref. 64). Recirculating bacterial-specific $T_H2$ memory cells may serve this purpose in airway tissues, mitigating the inflammatory responses against low-level bacterial incursions across inflamed airway epithelial barriers that may be a relatively common occurrence. In this regard, it should be noted that bacterial-specific IgE concentrations increase in the wake of viral-associated asthma exacerbations[60], which is consistent with 'boosting' by an incoming bacterial antigen.

The question of why IgE against *Haemophilus* and *Streptococcus* do not apparently provoke mast-cell–mediated inflammation may reflect the fact that the relevant antigens are only released as membrane-associated complexes that are accessible to phagocytes but are unsuitable for FcεR1 crosslinking. In contrast, IgEs against *Staphylococcus* superantigens that are secreted in a soluble form associate strongly with airway inflammation in the same children (reviewed in ref. 64).

## New opportunities for prevention and treatment of asthma

Efforts over the last 20 years to develop improved therapeutics for atopic asthma have focused primarily on $T_H2$ effector mechanisms in subjects with established disease. These include biologics and small-molecule antagonists specific for $T_H2$ cytokines and the IgE antibody itself. Many of these drugs are highly effective at blanketing their respective targets, but in the contexts in which they have been tested in clinical trials, none have provided evidence of long-term disease modification. Part of the problem is that the pathological processes described in **Figure 1** become progressively less reversible over time. Additionally, it is becoming apparent that subphenotypes of established asthma exist that vary subtly with respect to the relative contributions of different inflammatory pathways to pathogenesis[65,66]. We argue, however, that an equally crucial issue is the lack of alignment of therapeutic strategies with the growing body of epidemiological and clinical information on how patterns of disease expression evolve over time under the influence of different classes of environmental triggers and, in particular, how interactions between the ensuing pathological processes contribute to symptom severity at different stages of disease. Bringing these issues to the forefront of the considerations regarding to target identification and clinical trial design provides new perspectives on drug development in asthma that might logically lead to new approaches to treatment and, more crucially, to asthma prevention (**Box 2**). Prominent among these approaches are a series of strategies based on using existing drugs in nonstandard clinical contexts that are outside the current treatment paradigms. From these we nominate below a high-priority list of treatment options that, based on available safety and efficacy data, are amenable to immediate testing.

**Treatment of established atopic asthma.** The most crucial targets in established asthma are the acute exacerbations triggered by a virus and/or aeroallergen. Current clinical practice involves the use of inhalers to relieve symptoms of airway narrowing together with nonspecific anti-inflammatory drugs (typically corticosteroids). However, this approach is essentially empirical and takes no account of the efficiency of these drugs in controlling individual rate-limiting steps in the cycle that drives the exacerbation. The scheme in **Figure 2** and the proof-of-concept studies cited in **Box 2** provide a rationale for the use of anti-IgE and a topical IL-4/IL-13Rα antagonist in this context, specifically targeting AMDC-driven or FcεR1-facilitated aeroallergen presentation and the ensuing activation of $T_H2$ memory cells in the airway mucosa and RLNs; these are the key steps in the underlying inflammatory cascade, and blocking either step sufficiently early in the cycle (meaning close to symptom onset) may halt its progression toward bone marrow amplification. Later in the cycle, an IL-4/IL-13Rα antagonist given systemically may be the ideal candidate drug to truncate the downstream events in the bone marrow, notably the IL-4– or IL-13–dependent generation and programming of $CCR2^+$ lung-homing alternatively activated macrophages and the $Fc\varepsilon R1^{hi}$ replacements for airway dendritic cells.

**Halting the progression of atopic asthma during childhood.** Atopic asthma progresses through recognizable stages during childhood (**Box 1**), and there is growing interest in early identification of children already on a trajectory toward asthma to institute treatments to reduce the frequency of the inflammatory episodes driving disease progression and consolidation. The priority targets are as follows.

(i) Virus-associated exacerbations. The most persuasive recent data supporting this approach are from the US Inner City Asthma Consortium showing that children on monthly anti-IgE therapy had significantly reduced rates of exacerbations, particularly during the common cold season[67]. The seasonality of respiratory viral infections (especially rhinovirus) leaves open the strong possibility of 'common cold season only' prophylactic treatment of high-risk individuals with atopy as a viable public health measure. An additional possibility meriting testing is treatment during the common cold season with oral immune enhancer (IE) preparations[68], particularly those can stimulate mucosal regulatory T ($T_{reg}$) cell activity[69,70], such as those described in **Box 2**.

(ii) Inhalant allergy: halting the atopic march. The burgeoning area of immunotherapy focuses primarily on 'curing' allergic diseases. However, in light of the evidence that during childhood one form of allergy can lead to another (shown in the link between allergic rhinitis and subsequent asthma in adults and children[71,72] and **Fig. 2**), early immunotherapy in appropriately selected subjects will also probably be useful for preventing disease progression, and this idea is supported by proof-of-concept studies on asthma prevention in children with rhinitis[73]. Notably, the key cellular process that is probably driving this example of the atopic march, namely mucosal dendritic cell trafficking, is highly sensitive to topical corticosteroids[74]; therefore, despite the failure of long-term topical steroid use by inhaler to ameliorate established wheeze[75], nasal steroids merit testing as an asthma prophylactic in the specific context of children with allergic rhinitis who are asthma free.

**The 'holy grail': primary prevention of asthma.** The ultimate goal is to identify high-risk subjects in the infancy to preschool age range and institute measures to subvert atopic and viral-associated inflammation early enough to block the initiation of disease. As noted in **Box 1**, the wheezy phenotype shows a high degree of plasticity at these early ages but becomes progressively less reversible thereafter, marking this period as a potentially ideal therapeutic window for long-term disease modification. The atopy pathway is already being tackled using prophylactic immunotherapy (**Box 2**). However, it is not yet possible to conduct trials for anti-IgE in this context because safety data is lacking for this age group. However, this agent merits testing as soon as these data become available in light of a recent report showing the quantitative nature of the relationship between aeroallergen-specific IgEs and LRIs in early asthma development. Notably, the strength of the synergism between IgEs and LRIs increased linearly with antibody titers across the full range of LRI frequencies, even at IgE concentrations below the conventional sensitization thresholds.

Tackling the viral pathway in this young age group has been problematic. Whereas an effective antibody treatment for RSV exists (**Box 2**), it has not yet been made available for testing in this clinical context, despite strong support (which we endorse) from senior investigators in this field[16]. Alternative approaches include the use of preparations from the IE class, given its potential for reducing both infection frequency and intensity in preschoolers[68], and, potentially, type 1 IFNs.

## Future priorities

The cycles of episodic airway inflammation (**Fig. 2**) are driven by migrating $T_H2$ memory cells and myeloid cells responding to chemokine gradients through specific receptors such as CCR2 and the chemoattractant receptor-homologous molecule expressed on $T_H2$ cells ($CRT_H2$); targeting these players should be given high priority. Slightly over the horizon, rapid developments in relation to the airway microbiome are already creating expectations that antibacterials will soon join antivirals on the asthma agenda, despite justifiable concerns related to bacterial drug resistance. In our view, the key challenge is not the production of safe drugs that are effective against their designated targets (this will undoubtedly be achieved) but rather is to ensure that the clinical testing programs do justice to the upstream research and development efforts that led to their production. Achieving this for asthma will require a more disciplined approach to drug testing strategies than has been used in the past,

## BOX 2 The development of rational strategies for asthma treatment and prevention

### Respiratory infection resistance

**Antivirals**. A monoclonal antibody (mAb) for the treatment of RSV is available and has been shown to markedly reduce severe infections in infants[84].

**Orally delivered immune enhancers (IE)**. This area is dominated by probiotics, and this approach has the potential for the mitigation of respiratory infection severity in children[85]. A related class of therapeutics derived from mixed bacterial extracts has been used extensively to enhance infection resistance in patients with chronic respiratory diseases, for example, chronic obstructive pulmonary disease[86,87] and has been shown more recently to reduce the frequency and intensity of respiratory infections in young children[68,88].

**Type 1 IFN**. The use of IFN therapy for the prevention of viral infection has a checkered history, particularly in terms of side effects. In a recent development, a nasal spray containing low-dose recombinant IFN-$\alpha$-2b reduced influenza, parainfluenza and adenovirus infections in military recruits[89].

### Airway inflammation

**A topical antagonist to IL-4/IL-13R$\alpha$.** Studies with an aerosolized antagonist reported significant reduction in the $T_H2$ cell–dependent late-phase response to aeroallergen challenge in individuals with atopic asthma[90].

**MAb to IgE.** An mAb to IgE has been tested in a variety of clinical contexts since its initial release over 10 years ago, but the clinical effects achieved in conventional trial designs have been modest; recent studies have shown that continued use of these mAbs can reduce the frequency of exacerbations[67,91,92].

**$T_{reg}$ cell stimulants.** An unexpected recent finding in recent preclinical studies in rodent asthma models suggests that a major target for one class of the immune enhancer preparations is the expansion of mucosal-homing $T_{reg}$ cell population(s) that dampen airway inflammation[69,70]. This may explain their effects in reducing the clinical symptoms associated with respiratory infection[68].

### Myeloid cell activation in bone marrow

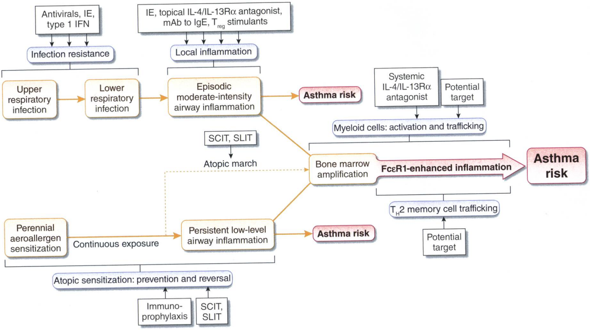
**A systemic antagonist to IL-4/IL-13R$\alpha$.** The agent detailed in reference 90 was equally potent in reducing the asthma late-phase response when delivered systemically in a form that would be bioavailable in bone marrow (and is also probably effective in airway RLNs).

### The atopy pathway

The concept of immunoprophylaxis (primary prevention of sensitization) in infants and young children with oral or sublingual allergen is currently being tested in trials sponsored by the US National Institutes of Health; extensive literature exists on immunotherapy for sensitized children and adults, in particular, proof-of-concept studies showing that desensitization of children with rhinitis who are allergic to pollen using subcutaneous immunotherapy (SCIT) prevents the subsequent development of asthma[73] and studies on recently developed sublingual immunotherapy (SLIT[93]).

### Gaps in the armor

Currently unavailable but highly relevant are drugs targeting the trafficking of myeloid and $T_H2$ cells. Also, as noted previously, this inflammatory cycle also has the potential for progression to a later phase that involves autocrine production of IL-13 by alternatively activated macrophages that may persist beyond viral clearance[29], and this cycle is potentially targetable with an mAb to IL-13 (ref. 94).


Antivirals, IE, type 1 IFN
IE, topical IL-4/IL-13Rα antagonist, mAb to IgE, Treg stimulants
Infection resistance
Local inflammation
Upper respiratory infection
Lower respiratory infection
Episodic moderate-intensity airway inflammation
Asthma risk
Systemic IL-4/IL-13Rα antagonist
Potential target
Myeloid cells: activation and trafficking
SCIT, SLIT
Atopic march
Bone marrow amplification
FcεR1-enhanced inflammation
Asthma risk
TH2 memory cell trafficking
Potential target
Perennial aeroallergen sensitization
Continuous exposure
Persistent low-level airway inflammation
Asthma risk
Atopic sensitization: prevention and reversal
Immuno-prophylaxis
SCIT, SLIT

with a sharper focus on the clinical context within which individual targeted mechanisms contribute to symptoms at different stages of disease. In terms of existing drugs, specifically those targeting IgE and IL-4/IL-13R$\alpha$, we have only scratched the surface with respect to their optimal clinical use in asthma treatment, particularly from the perspective of preventing progression to a chronic state.

### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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nature medicine

# Therapies for allergic inflammation: refining strategies to induce tolerance

Cezmi A Akdis

**Current therapies for asthma and allergy are relatively safe and effective at controlling symptoms but do not change the chronic course of disease. There is no established method to prevent asthma and allergy, and major unmet needs in this area include the better control of the severe forms of these diseases and the developments of curative therapies. Two major therapeutic strategies for asthma and allergy are currently being developed, and I here discuss the advances and challenges for future therapeutic development in these two areas. The first approach, allergen-specific immunotherapy, aims to induce specific immune tolerance and has a long-term disease-modifying effect. The second approach is the use of biological immune response modifiers to decrease pathological immune responses. Combination strategies using both of these approaches may also provide a route for addressing the unmet clinical needs in allergic diseases.**

Allergic inflammation is caused by the development of an allergen-induced immune response and can lead to several diseases, including asthma, allergic rhinoconjunctivitis, anaphylaxis, urticaria and atopic dermatitis[1]. It is now becoming clear that allergic diseases are complex disorders and that there are several disease variants caused by different underlying cellular and molecular mechanisms. A limited knowledge of the pathophysiology of these disease subgroups has been the greatest obstacle to identifying consistent correlations between genes, the environment and the different disease subgroups. Although there are several clinically relevant phenotypes for asthma, atopic dermatitis and urticaria, these phenotypes might not provide any insights into the mechanisms that underpin the diseases. It is now thought that some clinical trials may have previously been unsuccessful because they were performed without attempting to classify patients into subgroups defined by distinct pathophysiologies, namely 'endotypes'[2]. In asthma, phenotypes describe clinically relevant characteristics but do not necessarily give insights into the underlying pathological mechanisms of the disease. In contrast, asthma endotypes describe disease subtypes on the basis of cellular and molecular pathogenic mechanisms[3]. In addition, the results of large-scale genetic studies, particularly studies of asthma and atopic dermatitis, may have been inconclusive because of the bulk patient selection approaches used in many of these studies (**Box 1**).

Despite substantial improvements in treatments for asthma and allergy, approximately 5–10% of all people with these disorders have inadequately controlled severe persistent asthma or severe atopic dermatitis. These individuals use a large proportion of public health resources devoted to the treatment of asthma and allergy, and new, more effective therapies are urgently required. The possibility of curing allergic diseases is an essential issue for research because the medications currently used to treat these diseases, such as antihistamines, leukotriene receptor antagonists and glucocorticoids, only temporarily relieve symptoms by suppressing inflammation[4]. However, a long-term cure for allergic diseases can be achieved through the use of allergen-specific immunotherapy (allergen SIT), which has a disease-modifying effect and might also lead to decreased requirements for anti-inflammatory and symptomatic medications[5,6]. The basic principle of allergen SIT is to induce immune tolerance to allergens through multiple cellular and molecular mechanisms by administering repeated, increased doses of the causative allergen. Almost all of the immune tolerance mechanisms involved in responses to SIT are currently being targeted by drug discovery programs.

Unmet needs in treatment and advances in molecular biology and immunology have also spurred the development of new biological immune response modifiers to treat allergy and asthma. Such biological modifiers (biologicals) include therapeutic antibodies, soluble receptors, cytokines and small molecules, as well as combinations of these agents that can target effector molecules at various points in the immune and inflammatory pathways on different immune cells. So far, over 30 monoclonal antibodies have been approved for various clinical indications, particularly for autoimmune disorders, organ transplantation, infectious diseases and cancer. More than 300 biologicals are currently in clinical trials, and some researchers have suggested that there will be a switch from the use of chemicals to the use of biologicals in drug development within the next 10 years. Although this review will focus on the results from human clinical trials of such biological therapeutics, I also highlight promising therapeutic candidates from preclinical

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## BOX 1 Problems in drug development for the treatment of asthma

- Complexity of the whole disease spectrum
- Complexity of molecular mechanisms
- Limited biomarkers for subgrouping and endotyping
- Limited information on how to improve on existing therapies (for example, currently used inhaled steroid and β-adrenergic agonist combination therapy is effective and relatively inexpensive)
- Low patient adherence, which is a general problem for chronic diseases
- Small animal models are poorly predictive. Most drugs that are effective in mouse models have failed in clinical trials
- Individual outcomes are different, and different outcomes cannot be distinguished using a bulk approach
- It is not possible to study the combination of two new biologicals that may potentiate each other until one of them is approved; however, biologicals are unlikely to be effective when used alone

and mouse studies, which will need to be validated and tested in humans to see whether they fulfill their initial promise.

As there is evidence that allergen SIT might offer the possibility of a cure for asthma and allergic diseases, many research efforts are now concentrating on combining allergen SIT with new biological immune modifiers (**Table 1**). Such combination strategies could be advantageous over individual therapies in reducing side effects, providing synergistic effects that may allow for the treatment of persistent disease and providing more flexibility for treatments to be tailored according to different disease endotypes. Devising the separate treatment arms of the combination of allergen SIT and a biological immune modifier will pose substantial challenges, and we still have a long way to go before we can bring such combinations to the clinic.

### Mechanistic insights into immune tolerance to allergens

The concept of inducing immune tolerance has become crucial in the development of prevention and treatment strategies for many diseases in which dysregulation of the immune system has a key role, such as allergy, asthma, autoimmunity, organ transplantation and infertility. Immune tolerance to allergens is characterized by the establishment of long-term clinical tolerance[5,6]. In addition to the immune responses induced by various modes of allergen SIT, the development of healthy immune responses in beekeepers and cat owners who are exposed to high doses of allergens has been intensively studied to understand the mechanisms of allergen tolerance in humans[7,8]. Although several details of the tolerance process have yet not been elucidated, mechanisms involved include changes in the profiles of allergen-specific memory T and B cell responses and the production of specific antibody isotypes to skew the immune response in a noninflammatory direction, as well as decreased activation, tissue migration and degranulation of mast cells, basophils and eosinophils (**Box 2**).

**Rapid desensitization of mast cells and basophils by allergens.** Several mechanisms have been proposed to operate to ensure that mast cells and basophils are unresponsive to environmental proteins, even in the presence of specific immunoglobulin E (IgE). Notably, in patients, even after the first injection of allergen SIT, very early decreases in the susceptibility of mast cells and basophils to degranulation and decreases in systemic anaphylaxis are observed, although all of the treated individuals have high quantities of specific IgE. Although the underlying molecular pathways of this response are not yet elucidated, this mechanism seems similar to the one observed when these two immune-cell types are rapidly desensitized in anaphylactic reactions to drugs[9]. Anaphylaxis is associated with the release of inflammatory mediators from both mast cells and basophils, and successful hyposensitization alters the magnitude of the mediator release[10]. The release of these inflammatory mediators in low quantities (below the required dose for systemic anaphylaxis) may affect the threshold of activation of the mast cells and basophils[10,11].

**Regulatory T ($T_{reg}$) cells and peripheral T cell tolerance to allergens.** The induction of peripheral T cell tolerance, which is characterized primarily by the generation of allergen-specific $T_{reg}$ cells, is an essential step in allergen SIT[12,13] (**Fig. 1**). Peripheral tolerance is initiated by the cytokines interleukin-10 (IL-10) and transforming growth factor-β (TGF-β), which are produced in increasing amounts by the allergen-specific $T_{reg}$ cells as allergen SIT proceeds[12,13]. Roles have been shown for both IL-10–secreting type 1 regulatory T ($T_{reg}1$) cells or forkhead box P3 (FOXP3)-positive $T_{reg}$ cell subsets, suggesting that there is an overlap in the functions and characteristics of the cytokines and surface molecules of these inducible subsets of $T_{reg}$ cells in humans[14]. $CD4^+CD25^+$ $T_{reg}$ cells from atopic donors showed a reduced potential to inhibit $CD4^+CD25^-$ T cell proliferation, which is an indicator of peripheral allergen tolerance[15]. Allergen SIT has been shown to increase the number of $FOXP3^+CD25^+CD3^+$ cells in the nasal mucosa, and this increase associates with clinical efficacy and the suppression of seasonal allergic inflammation, supporting a putative role for $T_{reg}$ cells in the induction of allergen-specific

**Table 1 Features of and challenges for allergen SIT vaccines and biological immune response modifiers**

| Allergen SIT vaccine | Biological immune response modifier |
|---|---|
| Should induce long term allergen tolerance (curative) | Should decrease or suppress the pathological immune response |
| Should achieve clinical success in a short time with few doses | Persistent improvement should be achieved when the treatment is stopped |
| Should target individuals with an allergy to identified allergens | Should target inflammation that is refractory to conventional therapies |
| Biological and/or immunological markers should be identified for patient selection and assessment of which population should be targeted, when to start and stop therapy and how to follow the patients | Should be developed together with its specific biomarkers that give information on which population should be targeted, when to start and stop therapy and how to follow the patients |
| Use of multiple allergens at the same time is possible | Combined use of two or more biologicals is currently not possible |
| Molecular and cellular mechanisms of allergen or antigen tolerance have been mostly elucidated | There is a substantial amount of missing knowledge in the physiopathology of tissue inflammation |
| Was initiated 100 years ago and is clinically accepted | Relatively new approach with very few approved treatments |

## BOX 2 Unknown factors in the mechanisms of allergen SIT

- The molecular mechanisms of how $T_{reg}$ cells are generated *in vivo*
- How to develop improved adjuvants that can specifically induce $T_{reg}$ cells
- The *in vivo* life span of $T_{reg}$ cells induced by allergen SIT
- Whether there are there deleterious roles of $T_{reg}$ cells, such as immune tolerance to tumor antigens and chronic infectious agents
- The role of resident tissue cells in immune tolerance
- The molecular mechanisms of spontaneous healing, remissions and exacerbations of allergic disease
- The local tissue events that occur during SLIT and epicutaneous SIT
- Early molecular markers and predictors to decide whether to start or stop therapy and how to measure or predict therapeutic success
- The existence of differences in the mechanisms of high-dose and low-dose allergen SIT
- The mechanisms of long-term maintenance of allergen tolerance

tolerance[16]. A study investigating a wasp venom immunotherapy used human major histocompatibility complex (MHC) class II tetramers to monitor clinical allergen tolerance and showed that this therapy led to a switch in the frequencies of the antigen-specific T cells that produced certain cytokines. In response to the wasp venom immunotherapy, there was a marked decrease in the number of IL-4–producing T cells and an increase in the number of FOXP3$^+$ and IL-10–producing antigen-specific CD4$^+$ T cells[17]. Similarly, peptide immunotherapy in allergic asthma leads to decreased T helper type 2 ($T_H2$) cell responses as a result of IL-10–dependent peripheral T cell tolerance. Therapy with peptides of selected T cell epitopes from the Fel d 1 major cat allergen resulted in a suppression of T cell proliferation in response to other 'linked' T cell epitopes within the same allergen[18]. As has been shown in other models, such as in suppression of germinal center reactions and in intestinal lymphoid tissues, this T cell suppression can generally take place in both secondary lymphoid organs[19,20] and in the affected tissues[21].

The investigation of human high-dose allergen exposure models has also provided key insights into the nature of $T_{reg}$ cell responses in allergen tolerance. In nonallergic beekeepers and cat owners[7,8], $T_{reg}$ cells specific for the major allergens in bee venom and cat saliva represent the major T cell subset present in these healthy individuals. These $T_{reg}$ cells use numerous suppressive mechanisms, including the involvement of IL-10, TGF-β, cytotoxic T lymphocyte antigen 4 (CTLA4) and programmed death 1 (PD1)[7,22]. Supporting these findings, FOXP3 expression negatively correlates with the amount of IgE, eosinophilia and interferon-γ (IFN-γ), and the ratio of FOXP3$^+$ T cells to total CD4$^+$ T cells is significantly lower in individuals with asthma or atopic dermatitis compared to healthy individuals[23]. In addition, the expression of FOXP3 correlates with the suppressive capacity of $T_{reg}$ cells[24].

Studies of $T_{reg}$ cells in allergic individuals from other populations have also highlighted the key role of these cells in allergen tolerance. The induction of mucosal tolerance against dietary antigens is associated with the development of CD4$^+$CD25$^+$ $T_{reg}$ cells. Children who develop clinical tolerance to milk have decreased peripheral blood mononuclear cell proliferation in response to bovine β-lactoglobulin as a consequence of an increased number of circulating CD4$^+$CD25$^+$ $T_{reg}$ cells[25]. In allergic children, the number of $T_{reg}$ cells increases during the pollen season, and these $T_{reg}$ cells may have a role in the control of other T cell subsets that are activated by pollen allergens[26]. Both healthy and allergic individuals produce all three types of allergen-specific subsets of T cells, namely $T_H1$, $T_H2$ and $T_{reg}1$ cells, but in different proportions. Accordingly, a change in the balance between

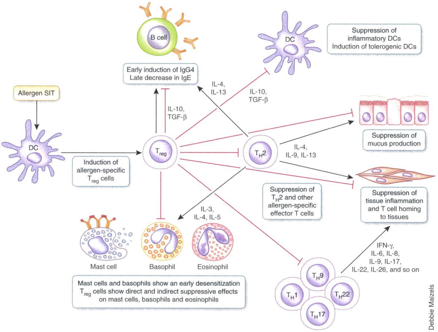

Allergen SIT
DC
Induction of allergen-specific $T_{reg}$ cells
$T_{reg}$
B cell
Early induction of IgG4
Late decrease in IgE
IL-10, TGF-β
IL-4, IL-13
IL-10, TGF-β
DC
Suppression of inflammatory DCs
Induction of tolerogenic DCs
$T_H2$
IL-4, IL-9, IL-13
Suppression of mucus production
Suppression of $T_H2$ and other allergen-specific effector T cells
Suppression of tissue inflammation and T cell homing to tissues
IL-3, IL-4, IL-5
Mast cell
Basophil
Eosinophil
Mast cells and basophils show an early desensitization
$T_{reg}$ cells show direct and indirect suppressive effects on mast cells, basophils and eosinophils
IFN-γ, IL-6, IL-8, IL-9, IL-17, IL-22, IL-26, and so on
$T_H9$
$T_H1$
$T_H22$
$T_H17$
Debbie Maizels

**Figure 1** Mechanisms of long-term immune tolerance obtained by treatment with allergen SIT. After the first administration of the SIT vaccine, there is an early decrease in mast cell and basophil degranulation and a decreased tendency for systemic anaphylaxis resulting from early desensitization. Allergen-specific $T_{reg}$ cells are then generated, and there is a suppression of allergen-specific $T_H2$ cells and other effector cells. Because of the immune tolerance of $T_H2$ cells, they can no longer contribute to IgE production, endothelial cell activation, $T_H2$ cell homing to tissues, mucus production by the epithelium or tissue migration, priming and survival of mast cells, eosinophils and basophils. IL-10 and TGF-β directly and indirectly regulate B cells and effector cells. Other T cell subsets, such as $T_H1$, $T_H9$, $T_H17$ and $T_H22$ cells, are suppressed by $T_{reg}$ cells. Within the spectrum of changes in the immune system after allergen SIT, there is a relatively early increase in the amount of IgG4 and a late decrease in the amount of IgE. A substantial decrease in the allergen-specific IgE-to-IgG4 ratio occurs several months after allergen SIT. A decrease in the number of tissue mast cells and eosinophils and in the release of their mediators and a decrease in late-phase response is observed in the affected tissues (red lines, suppression; black arrows, induction). DC, dendritic cell.

$T_H2$ and $T_{reg}$ cells, particularly in the dominant allergen-specific T cell subset, may lead to either the development of allergy or the recovery from allergic disease[7,22].

**Allergen-specific IgE and IgG responses.** Instead of becoming fully tolerant to allergen SIT as T cells do, B cells show a decisive skew from producing IgE to IgG4 in response to allergen SIT[27]. Allergen SIT induces a transient increase in serum-specific IgE concentrations followed by a gradual decrease in these concentrations over years of treatment[28]. In contrast, the specific concentration of IgG4 in serum has a relatively early and rapid increase in response to allergen SIT, and this concentration continues to increase during the whole duration that SIT is administered. IL-10 produced by $T_{reg}$ cells potently suppresses allergen-specific IgE while increasing IgG4 production[12,29]. Therefore, in addition to inducing T cell tolerance, IL-10 regulates the production of specific Ig isotypes, directing the immune response toward a noninflammatory phenotype. However, this effect of IL-10 cannot explain the observed therapeutic effect of allergen SIT because the decrease in serum IgE concentration in response to SIT, occurs relatively late in the process and does not correlate with clinical improvement after treatment. The reason for the long time gap between the generation of $T_{reg}$ cells after SIT and any changes in IgE concentrations is thought to be related to the continued production of IgE by plasma cells residing in the bone marrow that have a very long lifespan[30].

An analysis of the regulation of IgG subtypes by allergen SIT indicated that there are increases in the plasma concentrations of allergen-specific IgG1 and, particularly, IgG4 in response to SIT, and that these increases are in the range of 10- to 100-fold[31]. IgG4 competes with allergens for binding to the IgE on the Fcε receptors of mast cells and basophils and thus acts as a blocking antibody that prevents the activation and degranulation of effector cells[32]. In addition, some distinct features of IgG4 suggest that it may have an anti-inflammatory role. IgG4 antibodies are dynamic molecules that exchange fragment, antigen binding (Fab) arms by swapping heavy-light chain pairs between IgG4 molecules with different specificities. This process results in the production of bi-specific antibodies with a substantially decreased capacity for crosslinking because they are functionally monomeric[33]. In addition, the IgG4 hinge region has specialized structural features that result in a lower affinity for certain Fcγ receptors, and IgG4 does not fix complement and can inhibit immune-complex formation by other antibody isotypes[34].

**Suppression of mast cells, basophils and eosinophils.** The thresholds for mast cell and basophil activation and decreased IgE-mediated histamine release are efficiently modulated by allergen SIT[35]. Several molecular mechanisms have been proposed for how $T_{reg}$ cells and anti-inflammatory cytokines affect mast cells and basophils. IL-10 suppresses IL-5 production through human $T_H2$ cells, reduces proinflammatory cytokine release from mast cells and downregulates eosinophil function and activity[36]. Furthermore, through direct contact between $T_{reg}$ cells and mast cells, $T_{reg}$ cells inhibit Fcε receptor I (FcεRI)-dependent mast cell degranulation[37]. Mast cells also seem to have a role in immune tolerance and are not simply enhancers of allergic inflammation; for example, mast cells downregulate allergic inflammation in ultraviolet light–induced skin injury and venom-induced tissue damage models in which IL-10 has a key role[38,39]. $T_{reg}$ cells have been reported to be suppressive in various models of eosinophilic inflammation, including inflammation induced by schistosome infection and asthma-like lung inflammation in mice[40]. In addition, decreased numbers of eosinophils and decreased concentrations of eosinophil chemoattractants and eosinophil mediators have been observed in the nasal mucosa as a long-lasting effect of allergen SIT[41].

## Vaccine development for allergen SIT and immunomodulation

Although it has been performed in the clinic for the past 100 years, allergen SIT faces several problems related to its limited efficacy, side effects, low patient adherence and the high costs resulting from the long duration (3–5 years) of treatment required. The main approaches to improve the efficacy and safety of vaccine-based allergen SIT can be classified into six groups (**Table 2**).

The first approach is targeting T cells to induce T cell tolerance and bypassing IgE binding to avoid IgE-mediated side effects[42]. The conformation dependence of B cell epitopes and the linearity of the amino acid sequence of T cell epitopes in the three-dimensional structure of an allergen have been targeted using allergen fragments, fusions, hybrids and chimeras[42–45]. The prototype of this approach is peptide immunotherapy that uses linear T cell epitope peptides[46–49]. All of these T cell–targeting approaches enable higher doses of allergens to be sequentially administered, which is required to induce T cell tolerance without risk of anaphylaxis[42,50].

The second approach is the use of recombinant allergens or their mixtures to partially reconstitute an allergen extract. A study that tried to reconstruct a native grass pollen allergen extract using a mixture of five recombinant allergens was effective in reducing symptoms and the need for symptomatic medication in patients with grass pollen allergy[31]. All treated subjects developed high allergen-specific IgG1 and IgG4 antibody responses. During the study, seven systemic reactions were observed in patients from a total of 1,479 injections. Relatively mild local side effects related to treatment were observed in 10.7% of the patients that were actively treated with the recombinant allergens and in 5.9% of the patients receiving placebo. There have been 15 clinical trials that used recombinant allergens and showed greater clinical efficacy of the allergens compared to placebo over the last decade. However, licensing all these products may not be cost effective, particularly for minor allergens that do not cause allergic reactions in a substantial number of people. Recombinant vaccines for grass pollen, birch pollen and house dust mites have been the major focus in these trials.

The third approach is to physically couple allergens to stimulators of the innate immune response. This area is open to the possibility of future developments, as there are infinite numbers of possible combinations owing to the existence of multiple immune stimulators and methods for coupling[51–55]. Notably, the time, intensity and tissue location of stimulation of the innate immune response by the allergen are key to the induction of tolerance or immune activation. For example, house dust mites can activate Toll-like receptor 4 (TLR4) in airway structural cells to induce asthma-like inflammation in mice[56]. However, it should also be noted that natural exposure to house dust mites and other aeroallergens involves exposure to innate immune response–stimulating substances that activate TLR4.

The fourth approach is varying the routes of vaccine administration. A meta-analysis of double-blind, placebo-controlled trials of sublingual immunotherapy (SLIT) indicated that SLIT showed clinical efficacy and had a treatment benefit of approximately half of that achieved by subcutaneous allergen SIT[57]. Sustained disease-modifying effects of this type of therapy have been shown in large-scale randomized, double-blind, placebo-controlled trials in adults and in children[5,58]. Although the magnitudes of the changes in most clinical

**Table 2 Vaccine development for allergen SIT**

| Type of vaccine or approach | Developmental status | Comments | References |
|---|---|---|---|
| Bypassing IgE and targeting T cells | | | |
| Fusion of major allergens and chimeric allergens | Effects in human cell cultures and mouse models | Major allergens or their fragments are fused and expressed as a single recombinant protein. T cell reactivity is preserved, and IgE binding is attenuated. Prevention of IgE production has been shown in mice | 43,44 |
| Hypoallergenic hybrid molecules | Effects in human cell cultures and mouse models | Hybrid proteins derived from the Der p 1 and Der p 2 allergens reduce IgE reactivity and induce greater T cell proliferation | 121 |
| Fragments of major allergens | A multicenter clinical trial was reported in 2004. The approach has not been pursued further | Use of fragments of a major allergen (Bet v 1) that do not bind IgE. IgE binding is attenuated, and T cell reactivity is preserved | 45 |
| Peptide immunotherapy | Several clinical studies have been performed. Long peptides (27 amino acids in length) have been associated with side effects. Short peptides have been shown to be safe and tolerable in people allergic to cats in a dose-ranging phase 2a clinical trial (NCT00867906) | T cell epitope peptides (Fel d 1, Api m 1) that do not bind IgE and induce T cell tolerance have been used in cat and bee venom allergies | 46–49 |
| Unrefolded native or recombinant allergens | Several ongoing clinical trials have reported promising results | Major recombinant allergens (Api m 1, Bet v 1) that are not properly refolded and lack their native conformation have been used. IgE binding is abolished and T cell reactivity is protected | 50 |
| Polymers of major allergens | A multicenter clinical trial was finalized several years ago, and this approach has not been pursued further | A trimerized major allergen (Bet v 1) has been used. Mast cell and basophil degranulation is attenuated, and T cell reactivity is preserved *in vitro* | 45 |
| Reconstitution of the natural extract with multiple recombinant allergens | | | |
| Mixtures of several major recombinant allergens | One clinical trial reported promising results. A double-blind, placebo-controlled, dose-response study is under evaluation | Phl p 1, Phl p 2, Phl p 5a, Phl p 5b, Phl p 6 were used in combination as a mixture of five recombinant grass pollen allergens. A reduction in symptoms and the need for symptomatic medication was observed in people who were allergic to grass pollen | 31 |
| Allergens coupled to adjuvants | | | |
| GpG oligonucleotide-conjugated allergens | A large multicenter clinical trial did not reach its endpoints | A TLR9-triggering CpG oligonucleotide fused to a major ragweed allergen Amb a 1 has been tested | 51 |
| Allergens coupled to virus-like particles | A rapid induction of high IgG antibody titers was observed in healthy human volunteers | Highly repetitive virus capsid-like recombinant particles coupled to the house dust mite major allergen Der p 1 have been tested | 52 |
| Carbohydrate-based particles | Effects shown in mouse models | Carbohydrate-based particles bound to the allergen rPhl p 5b induced strong antibody and cytokine responses | 53 |
| Hypoallergenic vaccine based on allergen-derived peptides fused to hepatitis B PreS antigen | Effects shown in mouse models | Recombinant fusion proteins showed reduced allergenic activity with lowered basophil activation. There was no IgE reactivity to the fusion protein | 54 |
| Monophosphoryl lipid A (MPL) formulated with allergoid | Clinical trials have reported safety and efficacy. A phase 3 study has been completed (NCT00414141) | $\mathrm{T_H}$1-inducing adjuvant monophosphoryl lipid A (MPL) facilitated short-term SIT together with a grass pollen allergoid | 55 |
| New routes of administration | | | |
| Intralymphatic vaccination | A clinical trial has reported safety and efficacy. A phase 3 study is ongoing (NCT01166269) | Allergen SIT vaccines are administered directly into inguinal lymph nodes with the aim of delivering high amounts of allergens into secondary lymphatic organs | 63 |
| Epicutaneous vaccination | A clinical trial in grass-pollen–induced rhinoconjunctivitis showed safety and efficacy. A phase 2 study is ongoing in children with a peanut allergy (NCT01197053) | High numbers of antigen-presenting cells (Langerhans cells) are delivered to a nonvascularized area. The method is safe, needle free and has the potential for self administration | 64 |
| Fusion with immune response modifiers | | | |
| Targeting FcγRII | Effects have been shown in human cell cultures and mouse models | Fusion of allergens to human Fcγ has been reported to inhibit allergen-induced basophil and mast-cell degranulation by crosslinking Fcγ and FcεRI receptors | 65,66 |
| Modular antigen translocation (MAT) vaccines | A clinical trial has been finalized and showed safety and efficacy with evidence of immune regulation | The coexpression of major recombinant allergens together with the transactivator of transcription (Tat) peptide and truncated invariant chain peptide can target antigens to the MHC II molecules in the trans-golgi compartment | 68 |
| Combined treatment with immune response modifiers | | | |
| Pretreatment with mAbs to IgE before SIT | Several investigator-initiated clinical trials are ongoing to reduce SIT-induced side effects and to enable relatively rapid dose increases and the use of relatively high doses of therapy | Significantly fewer systemic allergic reactions were observed, and more patients were able to reach the target maintenance immunotherapy dose | 69 |

and immunological parameters have been modest or no changes have been observed in response to SLIT, the immunological mechanisms of SLIT seem to be similar to those of subcutaneous allergen SIT. The reduced treatment benefits and modest changes in immunological markers observed with SLIT suggest that there is still room to improve this method of vaccine delivery. Multiple mechanisms of immune tolerance are induced by SLIT that involve $T_{reg}$ cells, IL-10 production and increased numbers of sublingual FOXP3-expressing T cells. In addition, increased concentrations of blocking IgG4 and of IgA antibodies that show inhibitory activity for IgE-facilitated binding of allergens to B cells have been observed after SLIT, similar to what is seen after subcutaneous allergen SIT[59,60]. Recently, allergen-specific FOXP3$^+$ $T_{reg}$ cells have been found in human lingual and palatine tonsils in humans, and these cells may participate in oral allergen tolerance and SLIT[61]. It is possible that sugar-modified antigens can be used to induce oral tolerance, and a C-type lectin receptor, SIGNR1 (also known as CD209b), has recently been shown to condition dendritic cells to induce tolerance in the gastrointestinal lamina propria in a model of food-induced anaphylaxis[62].

Recently the intralymph node and epicutaneous routes of vaccine delivery have been tested. Both these routes showed a similar efficacy as subcutaneous immunotherapy injections in the treatment of grass pollen allergy, but fewer applications and lower total doses of allergen were required using these two routes[63,64]. Intralymphatic vaccines have been shown to induce T cell responses that are associated with strong cytotoxic activity and IFN-γ production, which are key in long-term protection against viral infections and tumors.

The fifth strategy is the fusion of allergens to immune modifiers. FcγRIIb is an immune tyrosine-based inhibitory motif-containing receptor[65]. The coaggregation of FcεRI and FcγRIIb inhibits FcεRI signaling, so one strategy that has been tested in the treatment of allergy is the fusion of FcγRIIb to allergens to downregulate downstream allergen-specific immune responses. In a similar approach, the fusion of allergens to human Fcγ suppressed allergen-induced degranulation of basophils and mast cells by crosslinking Fcγ and FcεRI[65,66]. Recently, the major cat allergen Fel d 1 was cloned and expressed together with a human immunodeficiency virus protein, TAT-derived membrane translocation domain, and a truncated peptide of the invariant chain (modular antigen translocation (MAT)-Fel d 1)[67]. This MAT–Fel d 1 vaccine is efficiently internalized and potently presented to T cells by antigen-presenting cells, and the vaccine induces T cell responses at doses that are approximately 100 times lower than those at which the native allergens are present. In a double-blind, placebo-controlled clinical trial, the MAT–Fel d 1 vaccine in alum adjuvant was administered in three increasing doses (1 μg, 3 μg and 10 μg) into the inguinal lymph nodes of patients with cat allergies at 4-week intervals. The vaccine showed a good safety profile, and after treatment, individuals who were allergic to cats became clinically tolerant to nasal challenge of cat dander extract, which was observed in parallel with increased concentrations of serum IgG4 in these individuals[68].

In addition to physical fusion, conventional and new methods of allergen SIT may also be combined with immune-response modifiers. For example, a monoclonal antibody (mAb) to IgE combined with allergen SIT has been evaluated in several studies[69], and this combination treatment resulted in a significant decrease in the risk of anaphylaxis caused by rush immunotherapy (which uses rapid dose increases to reach the maintenance therapeutic dose as quickly as possible) and improved rescue medication scores (therefore decreasing the need for a rescue medication to suppress the symptoms) of allergen SIT with a good safety profile[69,70]. Combination strategies with biological immune-response modifiers are expected to substantially expand the treatment scope of allergen SIT. Although there has been some progress in improving the efficacy and safety of allergen SIT, which is the only approach currently being investigated that could cure allergic diseases, further modifications could still be made to this mode of therapy, which are hoped to improve the application of SIT to allergic diseases as well as to other diseases related to immune dysregulation.

## Biological immunotherapeutics in allergy and asthma

Developments in understanding the immunological mechanisms of allergy and asthma have enabled the identification of many potential therapeutic targets (**Box 3**). Because of the complexity and redundancy of the immune mechanisms that are involved in asthma and allergy, it is possible that the use of a single biological might be effective in specific subgroups of asthma and atopic dermatitis, such as for well-defined endotypes that can identify those individuals who will benefit most from a particular treatment. Therefore, many new treatments that target a single mediator or receptor are currently in clinical development or preclinical investigation (**Table 3**). However, given the complex clinical spectrum of these diseases, it is improbable that any of these biologicals alone will have a major effect in a clinical trial testing a population of individuals that are heterogeneous for the allergic disease being studied or for asthma who have not been classified into specific phenotypes or endotypes. When a new drug is developed to target a very specific molecular mechanism, it is expected that it will function only in a specific asthma endotype. It is anticipated that endotype-tailored treatment (known as 'stratified medicine') could lead to measurable improvements in the health economics of asthma, but more importantly, it may enable disease management to be optimized for individual patients. Currently, combinations of biologicals or the sequential use of two or more biologicals are being considered as viable therapeutic strategies, as reported recently in a study that used a treatment combining mAb to IgE and mAb to B cell CD20 (ref. 71). However, the combination of two biologicals is prohibitively expensive, and it is practically impossible to combine biologicals in a clinical trial setting before one of them gets full approval. This problem needs to be solved before combination strategies of two unapproved biologicals can be developed, which may take several years.

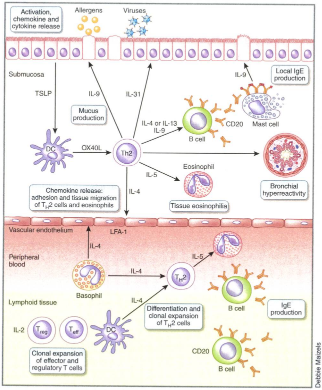
## Treatment strategies focused on IgE

Several clinical studies to test different humanized mAbs that bind to the Fc portion of the IgE molecule have been performed, and one of the mAbs tested, omalizumab, was shown to be an effective treatment in patients with poorly controlled, moderate to severe allergic asthma or allergic rhinitis[72–74]. Omalizumab decreases the serum concentration of free (unbound) IgE and the expression of the high-affinity IgE receptor FcεRI on several cell types[72]. Soluble immune complexes of IgE and mAb to IgE are subsequently cleared by the reticuloendothelial system. Omalizumab binds to the same site that IgE molecules use to attach to FcεRI and cannot crosslink cell-surface–expressed IgE[75].

Clinical studies of mAb to IgE have improved our understanding of the multiple roles of IgE in the development of the allergen-specific immune response and inflammation. Omalizumab reduces the expression of FcεRI on basophils, dendritic cells and monocytes within 7 d of administration[72]. Omalizumab treatment reduces the number of eosinophils in the blood of individuals with seasonal allergic rhinitis and asthma, as well as the number of sputum eosinophils

## BOX 3 Targets for new immunomodulatory drugs in asthma

- Epithelial cell activation and the proinflammatory cytokines and chemokines that they produce, which induce inflammation and contribute to $T_H2$ responses: TNF-α, IL-13, TSLP and IL-31, IL-33 (refs. 89,90,97,98,105,131,132, 134,137)
- Epithelial apoptosis and shedding in eczema and asthma[143,144]
- The $T_H2$ response: IL-4, IL-5, IL-9, IL-13, IL-25 and IL-33 (refs. 88–91,101,105,123,124,126–129,131–134)
- Eosinophilia: IL-5, IL-25 and IL-33 (refs. 91,105,127–129,133)
- Local and systemic IgE production: IL-4, IL-13, CD20 and IgE[88–90,119,123,124,126,131,132,145]
- Crosslinking of IgE receptor FcεRI on the surface of mast cells and basophils and their degranulation[146,147]
- Smooth muscle and myofibroblast activation and bronchial hyperreactivity: IL-4, IL-9, IL-13, IL-25, IL-33 (refs. 89,90, 101,105,131,132,134)
- Survival and reactivation of migrating inflammatory cells and their interaction with resident tissue cells and other inflammatory cells: IL-2, IL-4 (refs. 88,122–124,126)
- Cell migration and chemokines[110–112,139–141]
- Other effector T cell subsets, such as $T_H9$, $T_H17$ and $T_H22$ cells[148–150] (see illustration)


Activation, chemokine and cytokine release
Allergens
Viruses
Submucosa
TSLP
IL-9
IL-31
IL-9
Local IgE production
Mucus production
IL-4 or IL-13
IL-9
B cell
CD20
Mast cell
DC
OX40L
Th2
Eosinophil
IL-5
Chemokine release: adhesion and tissue migration of T<sub>H</sub>2 cells and eosinophils
IL-4
Tissue eosinophilia
Bronchial hyperreactivity
Vascular endothelium
LFA-1
IL-4
Peripheral blood
IL-5
IL-4
T<sub>H</sub>2
Basophil
Lymphoid tissue
IL-4
Differentiation and clonal expansion of T<sub>H</sub>2 cells
B cell
IgE production
IL-2
T<sub>reg</sub>
T<sub>eff</sub>
DC
Clonal expansion of effector and regulatory T cells
CD20
B cell

Debbie Maizels

in patients with asthma. In addition to its effects on mast cells and basophils that are related to decreasing the concentrations of free serum IgE, therapy with mAb to IgE causes a rapid decrease in FcεRI expression on the surface of dendritic cells, suggesting that circulating IgE regulates the expression of this molecule on dendritic cells[76]. A significant number of patients treated with omalizumab who had allergic asthma and rhinitis had a positive response to treatment and were able to decrease or discontinue their use of inhaled corticosteroids[73]. Studies of therapies using mAb to IgE in children have been limited, but a recent study showed that children, adolescents and young adults with atopic asthma living in the inner city who were treated with omalizumab had reductions in the number of asthmatic episodes, particularly if they were first sensitized to cockroach antigens[74]. However, two current caveats to the use of treatment with mAb to IgE are its unexpected efficacy in some cases without any reasonable explanation of the underlying molecular mechanisms, as well as considerations of cost effectiveness. In a recent study, treatment with omalizumab reduced histamine release from peripheral blood leukocytes stimulated with cat allergen *in vitro*; however, paradoxically, histamine release increased twofold after omalizumab-mediated basophil stimulation[77]. The cost effectiveness of omalizumab has been evaluated and has been shown to be low in patients with moderate to severe allergic asthma compared to the clinical benefits of the drug. Given this evaluation, one possible approach to use omalizumab is a relatively late treatment choice as an add-on therapy[78].

Although there have been no detailed double-blind, placebo-controlled studies on the subject, several reports have suggested that treatment with mAb to IgE is also beneficial for patients suffering from other IgE-related conditions, such as atopic dermatitis, peanut allergy, latex sensitivity, chronic urticaria or allergic bronchopulmonary aspergillosis. In allergic bronchopulmonary aspergillosis, omalizumab has been shown to eliminate the need for treatment with steroids[79]. Treatment with mAb to IgE has been reported to be effective in several cases of recurrent idiopathic angioedema[80] as well as in unprovoked anaphylaxis in patients with systemic mastocytosis[81]. In most patients with Churg-Strauss syndrome, a type of autoimmune vasculitis, severe asthma and upper respiratory symptoms persist, requiring continuous therapy. Treatment with mAb to IgE was shown to improve asthma and decrease the eosinophil blood count in individuals with this syndrome[82].

### Cytokine inhibitors

The inflammatory processes that underlie asthma and allergy are coordinated by a cytokine network. Modulating this network using new biological molecules has continuously been attempted in clinical trials for almost two decades. Some of these studies have been unsuccessful, and some trials have had to be discontinued, even for agents that have been approved for other indications, suggesting that there are substantial hurdles to be overcome for the clinical use of cytokine inhibitors to treat asthma and allergy. Some cytokines show redundancy in their ability to generate or suppress $T_H$ cell subsets and $T_{Reg}$ cells in addition to their roles in disease pathogenesis, which may indicate that agents targeting a single cytokine may not be effective. Despite this redundancy, therapies using cytokine inhibitors may provide a way to elucidate the role of individual cytokines in the pathogenesis of human disease, although it should also be acknowledged that the complexity of the inflammatory networks might make

**Table 3 Immune response modifiers that can be targeted for the treatment of allergy and asthma**

| Target | Molecule | Developmental Status | Comments | References |
|---|---|---|---|---|
| | | Approaches that target cytokines | | |
| IL-2 | mAb blocking IL-2 receptor α (CD25) (daclizumab) | Phase 2 has been completed, and efficacy has been observed for asthma | Inhibits the proinflammatory cytokine generation by IL-2R blockade on T cells. No negative effects have been observed on $T_{reg}$ cell generation *in vivo* | 122 |
| IL-4 | Humanized mAb blocking IL-4 (pascolizumab) | Phase 2 has been completed | Approaches that only targeted IL-4 were not effective, and developments have been halted | 123 |
| | Mutated IL-4 (pitrakinra): blocks IL-4Rα, which is common in IL-4 and IL-13 receptors | Reduced allergen responses in asthma and is in clinical trials. Phase 2 has been completed. Effective in a monkey model of asthma | Targeting of IL-4 and IL-13 at the same time showed some efficacy | 124 |
| | Blocking mAb to IL-4Ra, also blocks IL-13 (AMG-317) | Phase 2 has been completed, and some efficacy was observed | | 88 |
| | Inhaled oligonucleotide against IL-4Ra, also blocks IL-13 (AIR-645) | Inhibitory effects in allergen challenge. In clinical development. Phase 2 has been completed | | 125 |
| | Soluble recombinant human IL-4 receptor (altrakincept) | Phase 1 and 2 clinical trials showed efficacy for moderate asthma. A phase 3 trial did not confirm this efficacy | | 126 |
| IL-5 | Humanized blocking mAb to IL-5 (mepolizumab) | Effective in severe asthma with sputum eosinophilia and exacerbations in clinical trials. Phase 2 has been completed. Recruiting for phase 3 trial | Can be effective in an eosinophilic asthma endotype. Long-term inhibition of pulmonary eosinophilia and airway hyperresponsiveness was observed. Weak correlation with clinical symptoms, although peripheral eosinophila decreased | 127 |
| | Humanized blocking mAb to IL-5 (SCH-55700) | Reduced the number of circulating eosinophils in asthmatics and there was an improvement in baseline FEV1. Phase 2 is complete | | 91 |
| | IL-5a receptor mAb (MEDI-563) | Effective in asthma. Phase 2 is complete | | 128 |
| | Humanized blocking mAb to IL-5 (reslizumab) | Under development for the treatment of pediatric eosinophilic esophagitis and hypereosinophilic syndrome. Phase 2 has been completed for subjects with poorly controlled asthma | Reslizumab reduced intraepithelial esophageal eosinophil counts in children and adolescents with eosinophilic esophagitis. Improvements in symptoms were observed in all treatment groups, including placebo | 129,130 |
| IL-9 | Blocking mAb to IL-9 (MEDI-528) | Small effect in phase 2 studies | May only be effective in a subgroup of patients in which IL-9 has a dominant role in disease pathogenesis | 101 |
| IL-13 | Soluble IL-13Rα2 Fc fusion protein | Preclinical study in mice | IL-13Rα2 and IL-10 coordinately suppressed airway inflammation, airway hyperreactivity and fibrosis | 131 |
| | Blocking mAb to IL-13 (CAT-354) | Phase 1 clinical trial was completed investigating the pharmacokinetics. Phase 2 trial in patients with uncontrolled asthma despite optimal treatment has been completed | Reduced airway hyperreactivity, airway eosinophilia and esophageal eosinophilia in a mouse model of respiratory and esophageal inflammation induced by intratracheal human IL-13 | 89 |
| | Blocking mAb to IL-13 (lebrikizumab) | Improvement in lung functions in asthma. Phase 2 has been completed | Was effective in an endotype that had high levels of periostin expression | 90 |
| | Blocking humanized mAb to IL-13 (IMA-638) | Phase 1 and 2 trials have been completed | Dose-dependent inhibition of the allergen induced late responses and improved total nasal symptom scores in a subgroup with high late-phase nasal IL-13 concentrations at time of screening | 132 |
| IL-17 | Blocking mAb to IL-17 | Preclinical development for severe asthma in mice | | 102 |
| | Blocking mAb to IL-17RA | Preclinical development for severe asthma in mice | | 103 |
| IL-25 | Blocking mAb to IL-25 | Preclinical development in mice | Blocking IL-25 in an experimental model of allergic asthma prevented airway hyperreactivity, as well as a reduction in the IL-5 and IL-13 concentrations and eosinophilic inflammation | 133 |
| IL-31 | Blocking mAb to IL-31 | Tested in mice | Reduced scratching behavior in an atopic dermatitis model | 134 |
| IL-33 | Blocking mAb to IL-33 | Preclinical development in mice | IL-33 is an IL-1–like cytokine that can activate mast cells and $T_H2$ cells and can induce production of $T_H2$ cytokines | 105 |
| TSLP | Blocking mAb to TSLP, TSLPR-Ig | Preclinical development in mice | | 135 |
| TNF-α | Infliximab, chimeric mAb | Phase 2 for asthma has been completed | Targeting TNF-α has been considered to be a promising option, especially for the treatment of non-controlled asthma. Some studies have been stopped because of side effects, some studies have been completed with some efficacy, and some studies did not show any efficacy. May not be pursued because of unfavorable risk-benefit ratio. Essential to monitor and screen for infections | 97 |

(Continued)

**Table 3 (Continued)**

| Target | Molecule | Developmental Status | Comments | References |
|---|---|---|---|---|
| | Adalimumab, human mAb | Phase 2 study for asthma was withdrawn before enrollment | | 136 |
| | Golimumab, human mAb | Phase 2 for asthma has been completed | | 137 |
| | Etanercept, soluble TNF receptor fusion protein | Phase 2 study for moderate to severe and refractory asthma showed improved airway hyperresponsiveness, FEV1 values and overall asthma symptom scores. Phase 2 has been completed | | 98 |
| Light | Fusion protein blocking the receptor | Preclinical development in mice | | 99 |
| | | Cell adhesion and costimulation | | |
| LFA-1 | Humanized mAb against CD11a, LFA-1 (efalizumab) | Phase 2 has been completed.<br>Drug has been withdrawn from market | Reduced cellular infiltrate in atopic dermatitis lesions. Increased risk of infection, reactivation of latent and chronic infections and increased risk of progressive multifocal leukoencephalopathy | 138 |
| OX-40 ligand | Blocking mAb to OX40L | In preclinical development in mice | Inhibited $T_H2$-type immune responses induced by TSLP | 107 |
| | | Chemotaxis | | |
| CCR3 | Small-molecule antagonists | Toxicology problems in rats | | 139 |
| CCR4 | Blocking antibody (AMG-761) | Preclinical development in mice | | 140 |
| | Small-molecule antagonist (K-327) | Preclinical development in mice | | 141 |
| CXCR2 | Small-molecule antagonists (SCH527123) | Phase 2 studies have been completed | Effective at inhibiting neutrophil recruitment, mucus production and goblet cell hyperplasia in ozone-induced airway inflammation. Was well tolerated | 110 |
| DP1 | Selective agonist for DP1 receptor (BW245C) | Regulates immune and skin allergic responses in mouse models | The balance between DP1 and DP2 receptors is key in basophil responses | 111 |
| CRTH2 | Selective $CRT_H2$ antagonist (OC000459) | Phase 2 studies have been completed for asthma | Inhibitor of mast-cell–dependent activation of $T_H2$ lymphocytes and eosinophils | 142 |
| | | B cells | | |
| CD20 | Humanized mAb that binds to CD20 on B cells | Effective in atopic dermatitis in an investigator-driven clinical study | CD20 has a role in the regulation of human B cell activation, proliferation and differentiation | 119 |
| | | IgE | | |
| IgE | Humanized mAb that binds to Fc portion of human IgE (omalizumab) | Approved for the treatment of patients with persistent severe allergic asthma despite administration of optimized controller therapy | Safety and efficacy of this mAb in allergic conditions other than asthma and in pediatric patients below the age of 12 should be clarified | 72–74 |

these individual roles difficult to decipher. In addition, some of these biological modifiers could be possible candidates to augment the effects of allergen SIT in combination strategies.

**Strategies using mAb to IL-2.** It is generally thought that strategies that block IL-2 pathways could adversely affect $CD25^+FOXP3^+$ $T_{Reg}$ cell populations, which also rely on IL-2 signaling for their expansion and survival. In addition to promoting the proliferation and survival of recently activated effector T cells, IL-2 is also key in $T_{Reg}$ cell homeostasis, thymic development and suppressive function of $T_{Reg}$ cells. Daclizumab is a humanized monoclonal antibody that binds to the IL-2 receptor α chain CD25 on activated lymphocytes, inhibits IL-2–induced proliferation and reduces the production of $T_H2$ and $T_H1$ cell cytokines by activated T cells. A phase 2 study in patients with moderate to severe asthma showed that daclizumab improved pulmonary function, reduced asthma symptoms and the need for 'rescue' medication, increased the intervals between asthma exacerbations and reduced blood eosinophilia concentrations and the amount of serum eosinophil cationic protein[83,84]. The therapeutic benefit of daclizumab treatment in patients with more refractory asthma was more pronounced. The issue of whether blocking IL-2 signaling adversely affects $T_{reg}$ cell development and function was assessed in a heart transplantation study, which showed that daclizumab treatment did not interfere with $T_{reg}$ cell generation and had a beneficial effect on heart allograft survival[85].

**Strategies targeting IL-4.** IL-4 induces IgE isotype switching and promotes the differentiation of naive $CD4^+$ T lymphocytes into $T_H2$ cells, as well as the subsequent release of increased amounts IL-4, IL-5 and IL-13 by $T_H2$ cells. IL-4 may have a role in the expression of adhesion molecules and chemokines that promote the migration of $T_H2$ cells and eosinophils into tissues, as well as in the development of myeloid dendritic cells. Several clinical trials have evaluated the efficacy and safety of a recombinant human soluble IL-4 receptor (IL-4R) and mAbs to IL-4, but no significant improvements were noted in symptoms or asthma exacerbations using treatments with either of these two agents. These findings suggested that targeting IL-4 on its own does not seem to have clinical benefit and led to the discontinuation of the further exploration of therapies that target IL-4. One of the primary reasons why this approach failed could be the high redundancy of IL-4 and IL-13 signaling. Therefore, it may be necessary to block both IL-4 and IL-13 to see any efficacy for this type of therapy. The receptor of IL-4 and IL-13 consists of two chains, IL-4Rα (CD124) and the common γ chain. IL-13R consists of two subunits, IL-13Rα1 and IL-13Rα2, and IL-4 and IL-13 signaling occurs through the receptor complex type II that consists of the IL-4Rα and IL-13Rα (ref. 86).

A mutated IL-4 protein that inhibits the effects of both IL-4 and IL-13 through its ability to block IL-4Rα has been generated, and clinical studies testing therapies using this agent are currently under way[87]. A mAb to IL-4Rα that blocks both the IL-4 and IL-13 receptors may have the same potential as the mutant IL-4 protein for blocking the effects of both of these cytokines[88].

**Strategies using mAb to IL-13.** IL-13 is as crucial as IL-4 in the production of IgE, and studies have indicated that this cytokine induces airway hyperresponsiveness, epithelial activation, mucous production, chemokine secretion and changes in airway remodeling. Studies in mouse and monkey models of asthma have shown that treatment with mAb to IL-13 leads to a reduction in lung inflammation. Various humanized mAbs specific for human IL-13 have been, and are currently, in development. A phase 1 clinical trial showed that increasing single doses of intravenously administered mAbs to IL-13 in mild asthma were well tolerated at all doses, and there were no safety concerns associated with this treatment[89]. A recently reported phase 2 trial of a mAb to IL-13 (lebrikizumab) provided convincing evidence that this biological could be effective in treating certain disease subgroups that have been selected on the basis of a biomarker. Notably, this mAb to IL13 showed significant efficacy in a patient group that expressed high amounts of the protein periostin but did not have any effect in patients with low amounts of periostin, which is what would be predicted based on knowledge of the functions of periostin. IL-13 induces periostin, and periostin has a role in type I collagen synthesis, fibrosis and remodeling in the lungs[90]. Therefore, reducing the amount of free serum IL-13 or blocking IL-13 signaling in individuals with low amounts of periostin would not be expected to have any beneficial effect, as alternative pathogenic pathways that are not downstream of periostin are probably operating in these individuals.

**Strategies using mAb to IL-5.** Eosinophilia in the lung and circulation are hallmarks of asthma, and IL-5 is a key cytokine for eosinophil differentiation and survival. For this reason, there has been intensive focus on strategies using mAb to IL-5 for the treatment of asthma. A decrease in the number of serum eosinophils was noted in subjects with severe persistent asthma treated for 90 d with reslizumab, a humanized mAb to IL-5 (ref. 91). No significant sustained changes in forced expiratory volume in 1 s (FEV1), asthma symptom scores or the percentage of sputum eosinophils were noted with any dose of reslizumab. Studies in patients with mild atopic asthma showed that the humanized mAb to IL-5 mepolizumab decreased extracellular matrix protein remodeling and the percentage of airway eosinophils. Treatment with mepolizumab decreased serum eosinophilia by 100%, whereas it decreased airway eosinophils to a lesser extent, by 55%[92]. Similar to reslizumab, mepolizumab treatment did not affect the clinical measures of asthma, including airway hyperresponsiveness, FEV1 and peak flow measures. Recent trials of mAb to IL-5 in patients with prominent sputum eosinophilia and severe asthma that is refractory to existing therapies showed that the treatment significantly reduced asthma exacerbations[93]. Notably, in these trials, only 5% of the patients showed this clinical improvement and fit into a group that had eosinophils comprising greater than 3% of their total sputum cells, suggesting that it may be possible to define an asthma endotype that is responsive to mAb to IL-5, similar to the endotypes observed for periostin and lebrikizumab. If, similar to mAb to IL-5 and IL-13, a drug is only successful for a small percentage of affected individuals, it is now a matter of debate whether this factor would influence the likelihood of approval of the drug. In addition, treatment with mAb to IL-5 has shown promising results in patients with hypereosinophilic syndrome[94], nasal polyps[95] and eosinophilic esophagitis[96].

**Strategies to target tumor necrosis factor-α.** Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine that has been implicated in many aspects of the airway pathology in asthma, particularly in refractory and severe cases. Clinical trials have shown improvement in lung function, airway hyperresponsiveness and quality-of-life symptom scores and reduction in the exacerbation frequency in patients with asthma who were treated with mAb to TNF[97,98]. Treatment with etanercept, a soluble TNF receptor (TNFR) fusion protein, also improved airway hyperresponsiveness, FEV1 values and overall asthma symptom scores in a phase 2 study for moderate to severe and refractory asthma[98]. Although promising results have been obtained using these agents, because of their association with an increased risk of infection, particularly tuberculosis and malignancy, strategies that target TNF will probably not be enthusiastically pursued for general treatment of asthma.

The TNF superfamily consists of many membrane-bound and soluble proteins with proinflammatory effects in the innate and adaptive immune responses. A recent preclinical study showed that using a fusion protein to block the TNF ligand superfamily member 14, LIGHT, which is expressed on many immune cells such as activated T cells, monocytes and macrophages, reduced airway hyperresponsiveness, lung fibrosis and smooth muscle hyperplasia in mouse models of chronic asthma, despite showing little effect on airway eosinophilia in these models. LIGHT may be therapeutically targeted to prevent asthma-related airway remodeling, as LIGHT-deficient mice also showed an impairment in fibrosis and in smooth muscle accumulation[99]. However, given the safety concerns that already exist with therapies targeting TNF-α, it is probable that similar issues also exist for targeting LIGHT and other TNF-family molecules.

**Other approaches using cytokine inhibitors.** A number of other therapeutic approaches to target cytokines are in various stages of preclinical and clinical development and testing. A newly described population of T cells called $T_H9$ cells, which produce IL-9 and IL-10, have been proposed to have a role in allergic inflammation[100]. IL-9 is secreted by $CD4^+$ $T_H2$ cells, eosinophils, mast cells and neutrophils. It inhibits cytokine production by $T_H1$ cells, induces chemokine and mucous secretion by bronchial epithelial cells and promotes the proliferation of mast cells. In a recent clinical trial, an mAb to IL-9 did not to meet the clinical endpoints; however, it did show an acceptable safety profile, and findings from the trial were suggestive of clinical activity in subjects with mild to moderate asthma[101]. Approaches that target IL-17, IL-25, IL-31 or IL-33 may be useful in some endotypes of asthma and are currently in preclinical development[102–105]. However, positive results from preclinical studies do not necessarily mean that these approaches will work in the setting of human asthma, and in addition, specific endotypes that might be targeted by these cytokines have not been elucidated in humans.

Thymic stromal lymphopoietin (TSLP) is an essential cytokine for the initiation and development of allergic inflammation. Treatment with TSLP diverted airway tolerance against ovalbumin to $T_H2$ sensitization and inhibited the generation of ovalbumin-specific inducible $T_{reg}$ cells in an asthma-like mouse model[106]. A soluble TSLP antagonist, TSLPR immunoglobulin, was shown to reduce the severity of allergic disease by blocking TSLP signaling and by regulating pulmonary dendritic cells. The tumor necrosis factor receptor superfamily member 4 (OX40) ligand (OX40L) is a key regulator of TSLP-mediated $T_H2$ responses. Preclinical studies in a mouse model of asthma with an mAb that blocked OX40 signaling showed promising results, with substantial inhibition of the immune responses induced by TSLP in the lung and skin, including decreased $T_H2$ cell infiltration, cytokine secretion and IgE production. OX40L-blocking antibodies have also been shown to inhibit antigen-driven $T_H2$ inflammation in mouse and nonhuman primate models of asthma[107].

## Targeting cell adhesion, co-stimulation and chemotaxis

Chemokine receptors, which are G-protein–coupled receptors, can be therapeutically targeted by small molecules. In contrast, approaches to target cytokines and cytokine receptors using humanized blocking mAbs, soluble receptors and mutant cytokines are currently being investigated. As chemokines are involved in stimulating the migration of $T_H2$ cells and eosinophils into inflamed tissues, targeting these molecules and their receptors may be a useful strategy for the treatment of tissue inflammation in asthma and allergy. It is probable that a subset of chemokines are the key targets in asthma and are selective for eosinophils[108]. Selective eosinophil recruitment can be induced by chemokines that bind to chemokine (C-C motif) receptor 3 (CCR3) on eosinophils, such as eotaxin-1 (chemokine (C-C motif) ligand 1 (CCL11)), eotaxin-2 (CCL24), eotaxin-3 (CCL26), RANTES (CCL5) and MCP-4 (CCL13). Mice deficient in one of these chemokines have impaired eosinophil trafficking to the skin, airway and/or gut[109].

A key role for chemokine (C-X-C motif) receptor 2 (CXCR2) has been identified in asthma, chronic obstructive pulmonary disease and fibrotic pulmonary disorders. After CXCR2 inhibition, the angiogenesis and collagen deposition caused by lung injury is still observed, but the pulmonary damage induced by neutrophils, antigen or irritant-induced goblet cell hyperplasia is inhibited[110]. These features are common in inflammatory and fibrotic disorders of the lung. Clinical trials to evaluate small-molecule CXCR2 antagonists in chronic obstructive pulmonary disease, asthma and cystic fibrosis have been completed and have suggested that these molecules may be promising in the treatment of these conditions[110].

The prostaglandin and chemotactic receptors DP1 and $CRT_H2$ (also known as DP2) have key roles in the development and maintenance of allergy. Studies with DP1 and $CRT_H2$ antagonists have suggested that they could have a crucial role in modulating aspects of allergic diseases that are resistant to current therapies[111,112]. Prostaglandin D2 (PGD2) has a dominant role in mediating mast-cell–dependent activation of $T_H2$ lymphocytes, which is also mediated by $CRT_H2$ (ref. 112). PGD2 produced by mast cells might link the early and late phase allergic responses, and the antagonism of PGD2 is an attractive target for therapeutic intervention[111].

In people with asthma, a fraction of $CD4^+$ T cells express chemokine (C-X3-C motif) receptor 1 (CX3CR1), the receptor for CX3CL1. After allergen challenge, CX3CL1 expression is increased in the airway smooth muscle, lung endothelium and epithelium, and therefore this chemokine might be a target for immune modulation[113]. Consistent with this, wild-type mice treated with CX3CR1-blocking reagents and CXCR1-deficient mice show reduced inflammation in the lungs after allergen sensitization and challenge[113].

## Biologicals in atopic dermatitis

A subgroup of patients with severe atopic dermatitis requires systemic immunomodulatory treatment. Clinical studies of omalizumab, efalizumab (a humanized antibody to CD11a), infliximab, adalimumab and etanercept (agents that target TNF-α signaling) and rituximab have been performed with varying results and safety profiles. Efalizumab inhibits T cell activation and thereby impairs the recruitment of T cells into the skin. A retrospective analysis of 11 of the patients in the efalizumab study showed that only 2 had a positive outcome. Nine patients stopped treatment because of a progression of atopic dermatitis or a lack of any clinical effect[114]. In the study, in the individuals who showed disease progression after treatment with efalizumab, it was not clear whether this progression was a result of side effects of the drug or was a natural progression of the disease.

TNF-α plays a part in inflammation and keratinocyte apoptosis, leading to eczema in atopic dermatitis[115]. Infliximab, a chimeric mAb to TNF-α, has been used to treat sporadic cases of atopic dermatitis and has also been tested in an open, prospective pilot study of nine patients with atopic dermatitis that was refractory to conventional therapy[97]. The results from the treated patients and the pilot study suggest that infliximab can be used as an additional therapeutic option for refractory severe atopic dermatitis. Etanercept, which is a soluble TNF receptor, has been used as a treatment option in two patients with severe, chronic atopic dermatitis and led to clinically significant improvements in skin symptoms. These preliminary results suggest that etanercept therapy may be beneficial for atopic dermatitis, particularly in chronic variants associated with lichenification, which is characterized by a thickening and hardening of the skin as a result of the disease[116]. Similar to asthma, safety concerns for treatments that target TNF-α may be a caveat in their use for atopic dermatitis.

Omalizumab has been used in selected patients suffering from both atopic eczema and allergic asthma or from atopic eczema alone[117]. Improvements seen after omalizumab treatment in these patients seem to be a result of more immune regulatory mechanisms rather than of simple neutralization of IgE by omalizumab. Recently, a randomized, placebo-controlled, double-blind pilot study of omalizumab was performed in 20 patients, which showed that the drug did not improve the clinical course in patients with chronic atopic dermatitis[118]; however, it did improve the results of skin prick tests and atopy patch tests.

Rituximab is a chimeric mAb to CD20 that efficiently eliminates circulating B cells. B cells that have class switched to produce IgE have long been suggested to have a role in atopic dermatitis because highly elevated concentrations of serum IgE are commonly observed in patients with generalized extrinsic atopic dermatitis. A pilot study with six patients was conducted in which rituximab was intravenously administered in two doses of 1,000 mg given 2 weeks apart[119]. All six patients showed an improvement of their skin symptoms within 4–8 weeks of the start of treatment, suggesting that rituximab may be a promising treatment option for patients with severe atopic dermatitis. However, these initial results have not been confirmed in two patients treated with rituximab who did not show amelioration of disease symptoms after treatment[120]. Therefore, larger randomized studies will be required to more rigorously test the effects and mechanisms of immune regulation induced by omalizumab and rituximab in the treatment of atopic dermatitis.

## Conclusions and future perspectives

A better understanding of the molecular mechanisms involved in asthma and allergy is hoped to spur the development of new treatment modalities for these conditions. Currently, allergen SIT is the only available curative treatment for allergic diseases, as it can induce long-term allergen-specific immune tolerance using multiple mechanisms. Despite the benefits of this therapy for most treated individuals, not everyone improves; life-threatening side effects can occur, recovery may not be permanent and the duration of the treatment is long. Therefore, new vaccines, as well as reliable biomarkers to select patients who may have a good clinical response, are crucial. Notably, allergen-SIT–based curative approaches may also be promising for the prevention of allergic disease, although the challenges accompanying the prevention approaches might differ to those for treatment. The major challenges for prevention include the requirement for very early intervention, safety problems for pediatric usage of vaccines and the lack of early biomarkers to predict who will develop allergies and to which particular allergen.

There is also a strong rationale for the development of biological immune response modifiers. The future of this area of research should be exciting, as advances in immunology and bioengineering are being applied, allowing for the optimized design of biologicals to improve the clinical efficacy and the feasibility of clinical grade production of these agents. Patient-specific treatments that depend on the development of new diagnostic biomarkers that can discriminate between distinct disease endotypes are also on the horizon. The analysis of large datasets of adults and children with severe allergic diseases and asthma is now beginning to allow for the identification of distinct disease phenotypes. It will be crucial to identify biomarkers and use systems biology to predict clinical responses for various subphenotypes of the diseases. This area requires more research for personalized therapy to become feasible because only a small proportion of patients with severe allergic diseases (for example, asthma) are likely to respond to a single biological. In particular, the combination of immune response modifiers with allergen SIT might provide a way for efficient immunomodulation of allergic diseases. Moreover, a greater understanding of the underlying disease mechanisms in allergic diseases could bring the possibility of cure in a larger patient population closer to reality.

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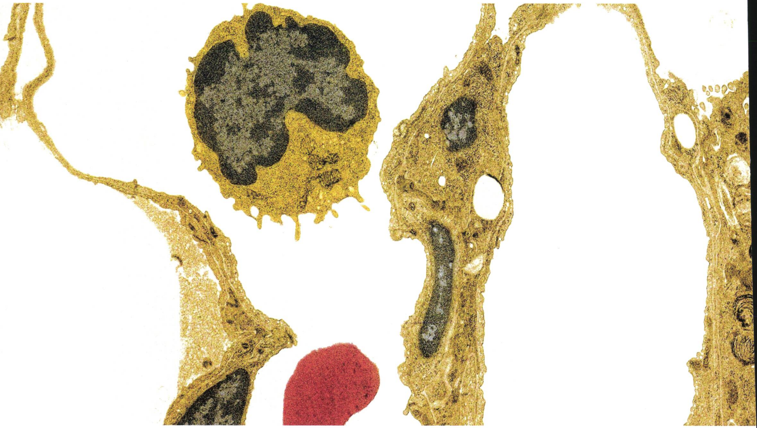

Focus on Asthma

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# Interleukin-25 induces type 2 cytokine production in a steroid-resistant interleukin-17RB$^+$ myeloid population that exacerbates asthmatic pathology

Bryan C Petersen[1], Alison L Budelsky[2], Alan P Baptist[3], Matthew A Schaller[1] & Nicholas W Lukacs[1]

**Interleukin-25 (IL-25) is a cytokine associated with allergy and asthma that functions to promote type 2 immune responses at mucosal epithelial surfaces and serves to protect against helminth parasitic infections in the intestinal tract. This study identifies the IL-25 receptor, IL-17RB, as a key mediator of both innate and adaptive pulmonary type 2 immune responses. Allergen exposure upregulated IL-25 and induced type 2 cytokine production in a previously undescribed granulocytic population, termed type 2 myeloid (T2M) cells. *Il17rb*$^{-/-}$ mice showed reduced lung pathology after chronic allergen exposure and decreased type 2 cytokine production in T2M cells and CD4$^+$ T lymphocytes. Airway instillation of IL-25 induced IL-4 and IL-13 production in T2M cells, demonstrating their importance in eliciting T cell–independent inflammation. The adoptive transfer of T2M cells reconstituted IL-25–mediated responses in *Il17rb*$^{-/-}$ mice. High-dose dexamethasone treatment did not reduce the IL-25–induced T2M pulmonary response. Finally, a similar IL-4– and IL-13–producing granulocytic population was identified in peripheral blood of human subjects with asthma. These data establish IL-25 and its receptor IL-17RB as targets for innate and adaptive immune responses in chronic allergic airway disease and identify T2M cells as a new steroid-resistant cell population.**

Current therapies for asthma focus on reducing the frequency and severity of exacerbations by attenuating bronchiole inflammation and airway hyperreactivity. The limited efficacy of treatments, such as inhaled corticosteroids, to reduce the inflammatory response in some chronic asthmatics illustrates the need for further research into mechanisms underlying the pathophysiology of asthma[1–3]. Three epithelial-derived type 2 inflammation associated cytokines, IL-25, thymic stromal lymphopoietin (TSLP) and IL-33, may represent targets for therapeutic treatment in severe asthma. IL-25, in particular, has been reported to enhance responses and further exacerbate allergic disease. In an original study[4], the systemic injection of IL-25 into *Rag*$^{-/-}$ mice induced type 2 cytokine expression and eosinophilia, demonstrating that T cell–independent mechanisms could drive type 2 inflammation.

The IL-17 family member IL-25 (IL-17E) regulates multiple aspects of mucosal immunity by promoting type 2 inflammation via production of IL-4, IL-5, and IL-13 (refs. 4–6). Pulmonary IL-25 is produced by eosinophils[7,8], mast cells and airway epithelial cells and stimulates asthma-like, allergic inflammation characterized by airway hyperreactivity, mucus production, airway eosinophilia and increased serum IgE[9]. The induction of these responses requires binding of a noncovalently bound IL-25 homodimer to the IL-17RA–IL-17RB heterodimer[10], of which IL-17RB represents an IL-25–specific moiety in the lung[11]. IL-25 is known to enhance T helper type 2 effector functions via Act1-dependent and TRAF6-dependent nuclear factor-κB activation[12–15]. Multiple cell types in the lung, including memory[16] and effector[14,16] T cells, invariant natural killer T cells[17], antigen-presenting cells and airway smooth muscle cells, have been shown to express IL-17RB, whereas eosinophils do not[7]. Several studies have also identified additional IL-25–responsive, type 2 cytokine–producing non-B, non-T (NBNT) cell populations[5,18–20], and a recent investigation reported increased expression of IL-25 and its receptor in the airways of individuals with asthma after allergen provocation[21].

This work reports that repeated allergen exposure upregulates both pulmonary IL-25 and IL-17RB in a mouse model of persistent allergic airway disease and induces the accumulation of a previously undescribed IL-4– and IL-13–producing IL-17RB$^+$ T2M population in the lung. Cytokine production in T2M cells is promoted by IL-25, and these cells are both pathogenic and steroid resistant. Moreover, IL-4– and IL-13–producing T2M-like cells are present in the peripheral blood of human subjects with asthma.

## RESULTS

### Chronic allergen drives type 2 cytokine production in myeloid cells

Several reports have linked IL-25 expression to the severity of allergic asthma[6,7,9,22,23]. C57BL/6J mice were sensitized via intraperitoneal and subcutaneous injection of cockroach allergen emulsified in incomplete Freund's adjuvant. The inflammatory response was localized to the lung via a series of six allergen challenges (four intranasal, and two intratracheal, given at 4-d intervals) starting 14 days postsensitization. The induction of type 2 cytokine expression following the pulmonary instillation of allergen (**Fig. 1**) was accompanied by

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increased mRNA expression of *Il25* and *Il17rb* (**Fig. 1c**) and tracked with the severity of the developing disease as depicted by histology (**Fig. 1a**). Neither *Ifng* (encoding interferon-γ), other IL-17 family members (**Fig. 1b,c**), *Il22*, *Il33* nor the IL-33 receptor *Il1rl1* was upregulated in our model of cockroach allergen challenge (data not shown), indicating that type 2 inflammation represents the dominant response induced by this model.

We have previously identified a pathologically relevant population of IL-17RB$^+$ myeloid cells with the capacity to produce IL-4 during chronic allergic airway disease[7]. Although CD4$^+$ T cells are present following antigen sensitization, the most numerous IL-17RB$^+$ IL-4– and IL-13–producing population in the lung was CD11b$^+$ myeloid cells (**Fig. 1d,e**). We also examined the capacity of innate Lin$^-$c-Kit$^+$Sca-1$^+$IL-17RB$^+$ NBNT cells to produce type 2 cytokines. Lin$^-$c-Kit$^+$Sca-1$^+$IL-17RB$^+$ cells made up a relatively rare population in the lungs of both naive and allergen-challenged mice (range 250–1,000) and were not higher in number following allergen sensitization (**Fig. 1d,e**). Few myeloid IL-4– and IL-13–producing cells were present in the lungs of naive mice, and the IL-17RB$^+$ cells preferentially produced type 2 cytokines. Myeloid IL-17RB$^+$ cells represented the major NBNT IL-4 and IL-13 cytokine-producing population in the lungs of allergen-challenged mice, outnumbering cytokine-producing CD4$^+$ T cells 68:1 (**Supplementary Fig. 1**).

Despite marked increases in IL-4– and IL-13–producing myeloid cells in environments with elevated levels of IL-25, not all pulmonary IL-17RB$^+$ myeloid populations seemed to produce these cytokines. Analysis of IL-4 and IL-13 production in IL-17RB$^+$ myeloid subsets, on the basis of levels of Gr-1 expression, allowed for the identification of two distinct IL-17RB$^+$ myeloid populations, Gr-1$^{\text{mid}}$ and Gr-1$^{\text{hi}}$ (**Fig. 1f**). A comparison of total cell numbers between naive and allergic mice identified significant increases in both Gr-1$^{\text{mid}}$ (**Fig. 1g**) and Gr-1$^{\text{hi}}$ (data not shown) subsets in the lungs of allergic mice; however, the IL-17RB$^+$CD11b$^+$Gr-1$^{\text{mid}}$ population produced IL-4 and IL-13, whereas the Gr-1$^{\text{hi}}$ population did not (data not shown). Isolation of the Gr-1$^{\text{mid}}$ subset by FACS identified a granulocytic population with a circular, partially segmented nucleus and relatively high nucleus-to-cytoplasm ratio (**Fig. 1h** and **Supplementary Fig. 2**). Next, we sought to investigate the involvement of IL-17RB in the production of type 2 cytokines in this granulocytic IL-17RB$^+$ population.

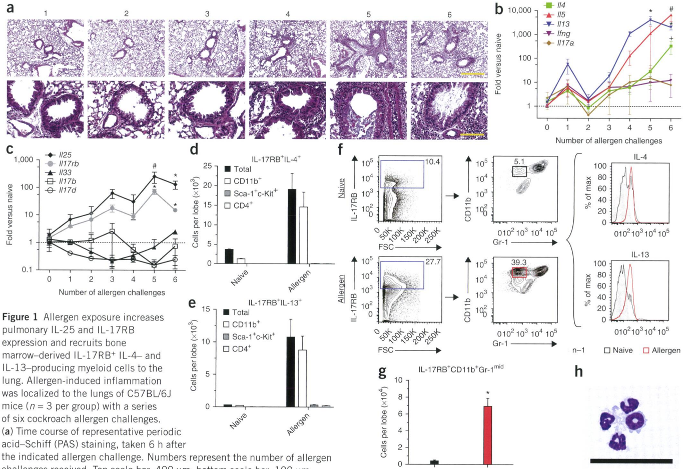


**Figure 1** Allergen exposure increases pulmonary IL-25 and IL-17RB expression and recruits bone marrow–derived IL-17RB$^+$ IL-4– and IL-13–producing myeloid cells to the lung. Allergen-induced inflammation was localized to the lungs of C57BL/6J mice ($n = 3$ per group) with a series of six cockroach allergen challenges. (**a**) Time course of representative periodic acid–Schiff (PAS) staining, taken 6 h after the indicated allergen challenge. Numbers represent the number of allergen challenges received. Top scale bar, 400 μm; bottom scale bar, 100 μm. (**b**) Time course of pulmonary *Il4*, *Il5*, *Il13*, *Ifng* and *Il17a* mRNA expression 6 h after the indicated number of allergen challenges. $^*P < 8.44 \times 10^{-5}$, $^\#P = 0.0008$, $^+P = 0.001$. (**c**) Thirty-two–day time course of *Il25*, *Il17rb*, *Il33*, *Il17b* and *Il17d* mRNA expression 6 h after the indicated number of allergen challenges. $^*P < 0.007$. (**d**,**e**) IL-4 (**d**) and IL-13 (**e**) production among IL-17RB$^+$ lung subsets from naive and allergen-sensitized C57BL/6J mice ($n = 5$ per group), as assessed by flow cytometry. (**f**) Representative flow plots of intracellular cytokine staining in IL-17RB$^+$CD11b$^+$Gr-1$^{\text{mid}}$ cells from naive and allergen-sensitized C57BL/6J mice. Gray: n-1 staining, black: naive IL-17RB$^+$CD11b$^+$Gr-1$^{\text{mid}}$, red: allergen-challenged IL-17RB$^+$CD11b$^+$Gr-1$^{\text{mid}}$. Numbers on the plots reflect the percentage of cells on each plot that are present within the gated area. (**g**) Pulmonary IL-17RB$^+$CD11b$^+$Gr-1$^{\text{mid}}$ populations following allergen sensitization in C57BL/6J mice. $^*P = 0.0026$. Results are representative of two independent experiments. (**h**) Morphology of myeloid cells isolated from the lungs of allergen-sensitized mice. Cells were sorted as CD11b$^+$Gr-1$^{\text{mid}}$ FcγR$^+$IL-17RB$^+$CD4$^-$CD8$^-$B220$^-$IL-7Rα$^-$Sca-1$^-$c-Kit$^-$ and stained with H&E. Scale bar, 50 μm. All data are presented as mean ± s.e.m.

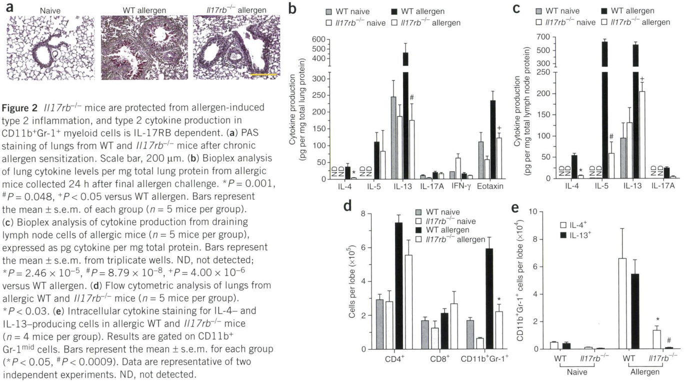


**Figure 2** *Il17rb*$^{-/-}$ mice are protected from allergen-induced type 2 inflammation, and type 2 cytokine production in CD11b$^+$Gr-1$^+$ myeloid cells is IL-17RB dependent. (**a**) PAS staining of lungs from WT and *Il17rb*$^{-/-}$ mice after chronic allergen sensitization. Scale bar, 200 μm. (**b**) Bioplex analysis of lung cytokine levels per mg total lung protein from allergic mice collected 24 h after final allergen challenge. $^*P = 0.001$, $^\#P = 0.048$, $^+P < 0.05$ versus WT allergen. Bars represent the mean ± s.e.m. of each group ($n = 5$ mice per group). (**c**) Bioplex analysis of cytokine production from draining lymph node cells of allergic mice ($n = 5$ mice per group), expressed as pg cytokine per mg total protein. Bars represent the mean ± s.e.m. from triplicate wells. ND, not detected; $^*P = 2.46 \times 10^{-5}$, $^\#P = 8.79 \times 10^{-8}$, $^+P = 4.00 \times 10^{-6}$ versus WT allergen. (**d**) Flow cytometric analysis of lungs from allergic WT and *Il17rb*$^{-/-}$ mice ($n = 5$ mice per group). $^*P < 0.03$. (**e**) Intracellular cytokine staining for IL-4– and IL-13–producing cells in allergic WT and *Il17rb*$^{-/-}$ mice ($n = 4$ mice per group). Results are gated on CD11b$^+$ Gr-1$^{\mathrm{mid}}$ cells. Bars represent the mean ± s.e.m. for each group ($^*P < 0.05$, $^\#P < 0.0009$). Data are representative of two independent experiments. ND, not detected.

## *Il17rb*$^{-/-}$ mice show decreased type 2 inflammation

To further explore the overall role of IL-17RB in allergic asthma, we sensitized *Il17rb*$^{-/-}$ mice to cockroach allergen as previously described and induced allergic airway disease with six allergen challenges over a 32-d period. The loss of the IL-25–specific receptor protected *Il17rb*$^{-/-}$ mice from allergen-induced inflammation (**Fig. 2**). *Il17rb*$^{-/-}$ allergic mice showed a marked reduction in peribronchial and perivascular inflammation, eosinophilic infiltrates and mucus production (**Fig. 2a**). Pulmonary expression of type 2 cytokines and of the eosinophil-associated chemokine CCL11 (Eotaxin) was significantly decreased in lungs of *Il17rb*$^{-/-}$ mice (**Fig. 2b**). *Il17rb*$^{-/-}$ draining lymph node cells re-stimulated with antigen produced significantly less type 2 cytokines than wild-type (WT) lymph node cells (**Fig. 2c**). Notably, a short-term model of allergen sensitization using only three allergen challenges revealed no phenotypic differences in *Il17rb*$^{-/-}$ mice (**Supplementary Fig. 3**), suggesting that IL-17RB is most relevant during more chronic allergic responses when IL-25 production is substantially increased.

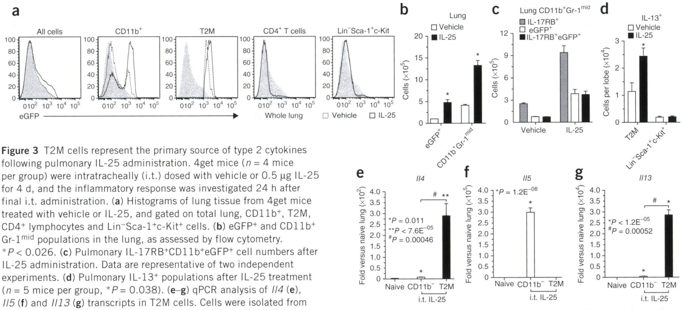
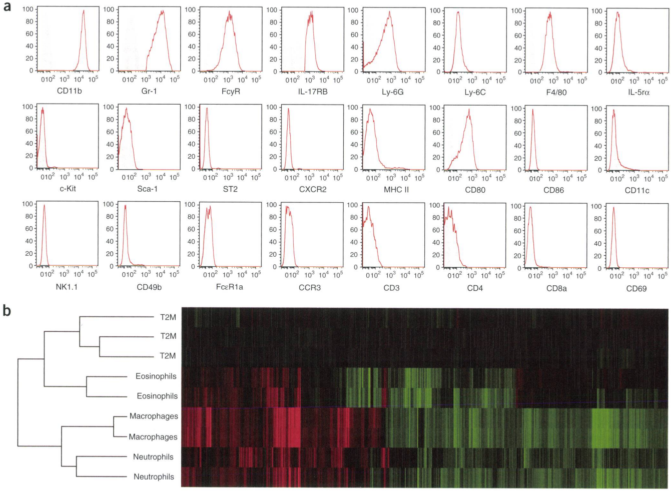


**Figure 3** T2M cells represent the primary source of type 2 cytokines following pulmonary IL-25 administration. 4get mice ($n = 4$ mice per group) were intratracheally (i.t.) dosed with vehicle or 0.5 μg IL-25 for 4 d, and the inflammatory response was investigated 24 h after final i.t. administration. (**a**) Histograms of lung tissue from 4get mice treated with vehicle or IL-25, and gated on total lung, CD11b$^+$, T2M, CD4$^+$ lymphocytes and Lin$^-$Sca-1$^+$c-Kit$^+$ cells. (**b**) eGFP$^+$ and CD11b$^+$ Gr-1$^{\mathrm{mid}}$ populations in the lung, as assessed by flow cytometry. $^*P < 0.026$. (**c**) Pulmonary IL-17RB$^+$CD11b$^+$eGFP$^+$ cell numbers after IL-25 administration. Data are representative of two independent experiments. (**d**) Pulmonary IL-13$^+$ populations after IL-25 treatment ($n = 5$ mice per group, $^*P = 0.038$). (**e**–**g**) qPCR analysis of *Il4* (**e**), *Il5* (**f**) and *Il13* (**g**) transcripts in T2M cells. Cells were isolated from C57BL/6J mice dosed with 0.5 μg IL-25 for 4 d ($n = 5$ mice per group) and plated in triplicate. T2M cells were isolated using MACS magnetic bead enrichment followed by FACS. mRNA was isolated from naive C57BL/6J mice, CD11b-depleted lung from IL-25–treated mice and T2M cells isolated from IL-25–treated mice. All data represent mean ± s.e.m.



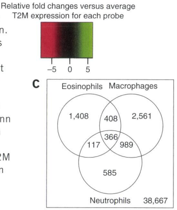


**Figure 4** Patterns of surface receptor expression and a comparison of microarray profiles define T2M cells as a distinct granulocytic subset. (**a**) Characterization of pulmonary T2M cells by cell surface receptor expression. 4get mice were challenged with intratracheal IL-25 to induce recruitment of T2M cells to the lung. T2M cells were identified by gating on CD11b$^+$Gr-1$^{\mathrm{mid}}$IL-17RB$^+$eGFP cells, and expression of various surface markers was then assessed. Gray shaded area: isotype, red line; T2M cells. Data are representative of two independent experiments. (**b**) Hierarchical clustering and heat map generated from microarray analysis of pulmonary T2M cells compared to eosinophils, neutrophils and macrophages. Colors illustrate fold changes among 1,880 probes for which T2M cells showed a minimum threefold difference in expression from two or more cell types and an average expression value of at least $2^6$ amplicons normalized to average T2M expression levels. (**c**) Venn diagram illustrating differences in locus expression between T2M cells and other myeloid populations. Within the Venn diagram, numbers reflect probes at which the indicated population differed from T2M cells. These numbers quantify the data presented in **b** and expand the data set to include probes that differed between T2M cells and only one other population. The same criteria to determine differences in probe expression (minimum threefold difference, with an average expression value of $2^6$) were applied. 366 is the number of probes at which T2M cell expression differed from eosinophils, macrophages and neutrophils. 1,408 is the number of probes at which T2M cells showed differences with eosinophils only. 408 is the number of probes at which eosinophils and macrophages showed differential expression from T2M cells. 38,667 is the number of probes whose expression did not differ between the four cell types. 1,880 probes differed between T2M cells and at least two of the comparison populations.

There was no detectable difference in total numbers of CD4$^+$ or CD8$^+$ T lymphocyte subsets between the lungs of allergen-sensitized *Il17rb*$^{-/-}$ and WT mice. However, *in vitro* re-stimulation experiments, as well as *in vivo* adoptive transfer studies with *Rag2*$^{-/-}$ mice lacking B and T cells demonstrated that CD4$^+$ T cell responses were altered in *Il17rb*$^{-/-}$ mice and that *Il17rb*$^{-/-}$ CD4$^+$ T cells could not efficiently transfer a type 2 immune response to the *Rag2*$^{-/-}$ recipients (**Supplementary Fig. 4**). Despite the ability of both WT and *Il17rb*$^{-/-}$ T cells to induce a type 2 inflammatory response, we observed a marked reduction in overall pulmonary inflammation and subsequent response to allergen in *Rag2*$^{-/-}$ recipients of *Il17rb*$^{-/-}$ T cells compared to WT recipients (**Supplementary Fig. 4**). This altered response affected the recruitment of other cells via a reduction in transcripts of allergen-associated chemokines such as *Tarc* (CCL17) (**Supplementary Fig. 4**). It also led to a significant ($P < 0.03$) decrease in myeloid infiltrates following allergen challenge, indicating that the absence of IL-17RB in CD4$^+$ T cells led to a systemic deficiency in inducing a type 2 cytokine–mediated inflammatory environment. These data were similar to findings published by several groups establishing a role of IL-25 in type 2 responses[9,10,16]. *Il17rb*$^{-/-}$ mice also

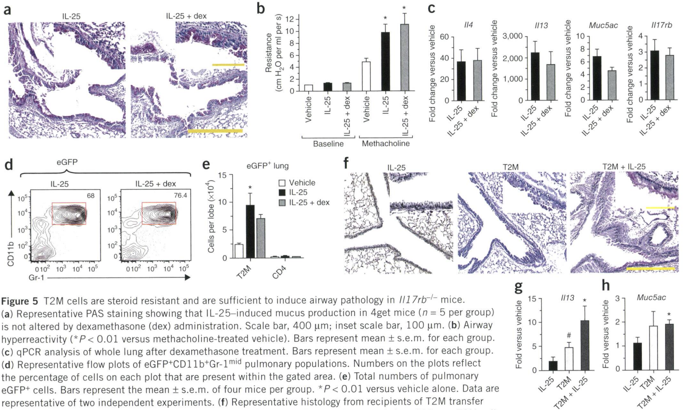


**Figure 5** T2M cells are steroid resistant and are sufficient to induce airway pathology in *Il17rb*$^{-/-}$ mice. (**a**) Representative PAS staining showing that IL-25–induced mucus production in 4get mice ($n = 5$ per group) is not altered by dexamethasone (dex) administration. Scale bar, 400 μm; inset scale bar, 100 μm. (**b**) Airway hyperreactivity ($^*P < 0.01$ versus methacholine-treated vehicle). Bars represent mean ± s.e.m. for each group. (**c**) qPCR analysis of whole lung after dexamethasone treatment. Bars represent mean ± s.e.m. for each group. (**d**) Representative flow plots of eGFP$^+$CD11b$^+$Gr-1$^{\text{mid}}$ pulmonary populations. Numbers on the plots reflect the percentage of cells on each plot that are present within the gated area. (**e**) Total numbers of pulmonary eGFP$^+$ cells. Bars represent the mean ± s.e.m. of four mice per group. $^*P < 0.01$ versus vehicle alone. Data are representative of two independent experiments. (**f**) Representative histology from recipients of T2M transfer stained with PAS, 24 h after final IL-25 administration. Scale bar, 400 μm; inset scale bar, 100 μm. T2M cells were isolated by MACS enrichment and FACS, and $2.0 \times 10^5$ cells were instilled into the airways of *Il17rb*$^{-/-}$ mice. Mice received four total treatments, consisting of 0.5 μg IL-25, T2M cells alone or IL-25 + T2M cells. (**g**) qPCR expression of IL-13 after T2M transfer ($^*P = 0.017$, $^\#P = 0.042$). Bars represent the mean ± s.e.m. (**h**) qPCR expression for the mucus-specific gene *Muc5ac* ($^*P < 0.026$). Bars represent the mean ± s.e.m. of four mice per group. Results are representative of three independent experiments.

showed significant reductions in the frequency of CD11b$^+$Gr-1$^{\text{mid}}$ myeloid cells per lobe that we previously identified as a source of type 2 cytokines in allergic mice (**Fig. 2d**). Intracellular cytokine staining of the CD11b$^+$Gr-1$^{\text{mid}}$ population verified that *Il17rb*$^{-/-}$ mice had a significant reduction in type 2 cytokine–producing cells relative to WT mice (**Fig. 2e**). Reduced cytokine production in lymph nodes, altered CD4$^+$ T cell function, a reduction in type 2 cytokines in the lungs and in CD11b$^+$Gr-1$^{\text{mid}}$ myeloid cells, and a marked decrease in pulmonary myeloid cell infiltrates indicated that multiple cell types were affected by the absence of IL-17RB.

## IL-25-induced inflammation is T2M cell dependent

We adapted a model of antigen-independent, IL-25–induced pulmonary inflammation[4] to directly assess the effects of IL-25 on type 2 cytokine–producing cells *in vivo*, thereby avoiding the confounding proinflammatory effects of antigen-specific activation. Recombinant mouse IL-25 was instilled into the airways of IL-4–IRES-eGFP (4get) mice. 4get mice express eGFP in cells in which the *Il4* promoter is transcriptionally active, and we used them to identify cells poised to produce type 2 cytokines. As has been reported previously[4], the intratracheal administration of IL-25 induced a type 2 inflammatory response, characterized by airway hyperreactivity, eosinophil infiltrates, mucus production and the upregulation of inflammatory genes, including *Il25* and *Il17rb* (**Supplementary Fig. 5**). IL-25 instillation induced the expression of eGFP in myeloid but not other cell subsets (**Fig. 3a**), with approximately 80% of eGFP cells being CD11b$^+$. The CD11b$^+$Gr-1$^{\text{mid}}$IL-17RB$^+$ subset, termed T2M cells to describe their propensity for type 2 cytokine production, showed particularly marked enrichment for eGFP expression. Whereas IL-25–treated mice showed no increase in other eGFP populations, IL-25 administration significantly increased CD11b$^+$Gr-1$^{\text{mid}}$ infiltrates (**Fig. 3b**). Among this population, all eGFP$^+$ cells were also IL-17RB$^+$ (**Fig. 3c**), indicating that IL-25 acts on IL-25–responsive myeloid cells in part by activating transcription at the *Il4* promoter. Flow cytometric analysis further confirmed that the predominant pulmonary source of IL-13 following IL-25 administration was T2M cells (**Fig. 3d**). Lin$^-$c-Kit$^+$Sca-1$^+$IL-17RB$^+$ cells, a population identified as a source of IL-25–induced IL-13 in the gut[19], were not altered by pulmonary IL-25 administration (**Fig. 3d**).

To verify that myeloid cells were producing type 2 cytokines in response to IL-25 administration, we isolated T2M cells from the lungs of IL-25–treated mice and assessed type 2 transcripts in this population. T2M cells exposed to IL-25 *in vivo* showed marked increases in *Il4* and *Il13* transcripts, whereas *Il5* was derived from a CD11b$^-$ cell population that remains ill-defined in our studies (**Fig. 3e–g**). In addition to the increase in pulmonary T2M cells that we observed after intratracheal IL-25 administration, we also identified T2M cells in the bone marrow of 4get mice after IL-25 treatments. The pulmonary instillation of recombinant IL-25 increased numbers of bone marrow T2M cells and induced IL-4 expression by the bone marrow T2M population (**Supplementary Fig. 6**). Thus, IL-25 exerts both local and systemic effects by increasing numbers of T2M cells in lung and bone marrow and by stimulating cytokine production in these populations.

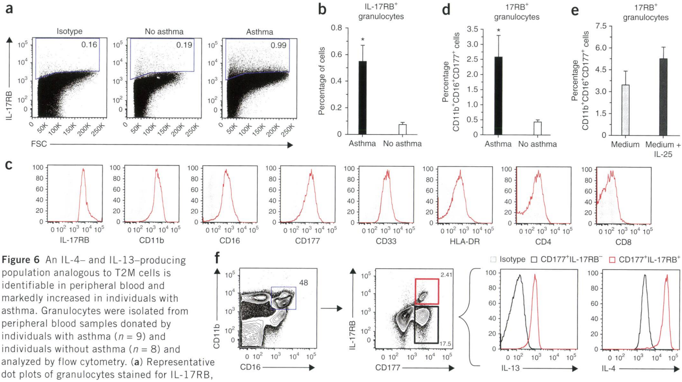


**Figure 6** An IL-4– and IL-13–producing population analogous to T2M cells is identifiable in peripheral blood and markedly increased in individuals with asthma. Granulocytes were isolated from peripheral blood samples donated by individuals with asthma ($n = 9$) and individuals without asthma ($n = 8$) and analyzed by flow cytometry. (**a**) Representative dot plots of granulocytes stained for IL-17RB, normalized to 400,000 events. (**b**) Percentage total IL-17RB$^+$ granulocytes isolated from peripheral blood of volunteer donors. Bars represent the mean ± s.e.m. for each group, $^*P = 0.0031$. (**c**) Representative histograms of IL-17RB$^+$ granulocytes from a donor with asthma indicate the majority of IL-17RB$^+$ granulocytes are CD11b$^+$CD16$^+$CD177$^+$. Cells were gated on total IL-17RB$^+$ cells and assessed for surface marker expression. (**d**) Percentage total CD11b$^+$CD16$^+$CD177$^+$IL-17RB$^+$ granulocytes from volunteer donors. Bars represent the mean ± s.e.m. for each group, $^*P = 0.023$. (**e**) Percentage total CD11b$^+$CD16$^+$CD177$^+$IL-17RB$^+$ cells from whole blood of volunteer donors with asthma, cultured *in vitro* for 2 h with or without 50 ng ml$^{-1}$ IL-25. Bars represent the mean ± s.e.m. (**f**) Representative intracellular cytokine staining for IL-4 and IL-13 from whole blood from a volunteer with asthma, cultured for 2 h with RPMI-1640. Numbers in the plots reflect the percentage of cells on each plot that are present within the gated area.

Pulmonary T2M cells are defined by a distinct combination of cell surface antigens (**Fig. 4a**). We observed similar expression patterns of cell surface antigens in T2M cells derived from other tissues, including spleen, bone marrow and peripheral blood (data not shown). T2M cells did not express the neutrophil-specific receptor CXCR2, nor did they express eosinophil protein markers such as IL-5rα and CCR3. FACS-isolated T2Ms did not produce detectable transcripts encoding myeloperoxidase, major basic protein or eosinophil peroxidase (data not shown), further supporting the claim that they are a separate granulocytic population. Microarray analysis of T2M cells compared to other isolated myeloid cell populations (**Fig. 4b**,**c** and **Supplementary Fig. 2**) provides further evidence that this population represents a distinct granulocytic subset most closely related to eosinophils.

## T2M cells are steroid resistant and pathologically relevant

To examine potential clinical implications of type 2 cytokine production in the T2M population, we next examined how steroid treatment affected IL-25–induced pulmonary inflammation. To focus on the T2M response, we treated 4get mice with IL-25 as in previous experiments, with or without dexamethasone. Histologic examination (**Fig. 5a**) and measurements of airway hyperreactivity (**Fig. 5b**) indicated that IL-25–induced responses were not significantly altered by dexamethasone. Quantitative PCR (qPCR) analyses demonstrated marked increases in the expression of type 2 cytokine genes, mucus genes and *Il17rb* that were not suppressed after dexamethasone administration (**Fig. 5c**). Flow cytometric analysis revealed equivalent numbers of IL-25–induced eGFP$^+$ myeloid cells in mice treated with or without dexamethasone (**Fig. 5d**,**e**). To verify that the dexamethasone treatment was effective, we examined splenic cell subsets (**Supplementary Table 1**). Dexamethasone significantly reduced the number of total splenocytes, with a specific reduction in CD4$^+$ and CD8$^+$ T cells as well as eosinophils, but it had no effect on splenic T2M cells. Overall, these data indicate that IL-25–induced T2M cells are resistant to high-dose glucocorticoid treatment.

To determine whether IL-17RB$^+$ T2M cells are sufficient to induce pulmonary inflammation, we isolated T2M cells from IL-25–treated mice and instilled them into the airways of *Il17rb*$^{-/-}$ mice together with recombinant IL-25. The transfer of T2M cells from IL-25–treated WT mice into *Il17rb*$^{-/-}$ recipients, coupled with instillations of IL-25, induced mucus production and inflammation in otherwise IL-25–insensitive *Il17rb*$^{-/-}$ mice (**Fig. 5f**). Recipients of T2M cells had significantly higher *Il13* transcript levels, and the transfer of T2M cells with IL-25 further upregulated expression of *Il13* (**Fig. 5g**) and the mucus-specific gene *Muc5ac* (**Fig. 5h**). In separate experiments, T2M transfer exacerbated the inflammatory response in WT recipients and increased *Muc5ac* transcripts to levels comparable to those observed in IL-25–treated WT mice (**Supplementary Fig. 7**). We observed a similar pattern of increased mucus-specific gene expression after the adoptive transfer of T2M cells into allergen-sensitized recipients (**Supplementary Fig. 7**). Therefore, in the context of high pulmonary IL-25 levels, T2M cells are sufficient to induce pulmonary inflammation, IL-13 expression and mucus production.

### T2M-like cells are increased in individuals with asthma

To assess whether a population analogous to T2M cells is present in humans and may be clinically relevant, we recruited volunteer subjects with asthma from the University of Michigan Asthma Clinic and compared their expression of IL-17RB in peripheral blood to that of nonasthmatic volunteers. Flow cytometric analysis identified significantly higher numbers of granulocytic IL-17RB$^+$ cells in subjects with asthma (**Fig. 6a**,**b**). The granulocytic IL-17RB$^+$ population concurrently expressed CD11b, CD16, and the Ly6 family member/myeloid progenitor/neutrophil marker CD177 (**Fig. 6c**). In addition, most IL- 7RB$^+$ cells were CD33$^+$, weakly expressed human leukocyte antigen-DR and were not CD4$^+$ or CD8$^+$.

Given the above findings, we focused our analysis on IL- 7RB$^+$ CD11b$^+$CD16$^+$CD177$^+$ cells and identified a significantly higher percentage of these cells in subjects with asthma (**Fig. 6d**) that was further elevated after *in vitro* stimulation with IL-25 (**Fig. 6e**). This IL-17RB$^+$ subset produced both IL-4 and IL-13, whereas IL-17RB$^-$ cells did not (**Fig. 6f**). Thus, a population with similar cell surface receptor expression and phenotype as mouse T2M cells can be identified in peripheral blood, is significantly elevated in asthmatics, and represents a source of both IL-4 and IL-13.

## DISCUSSION

IL-25 has been established as a regulator of type 2 inflammation, and multiple reports have described its ability to exacerbate inflammatory responses at mucosal epithelia, including those in allergic asthma. The present study used a mouse model of chronic allergic asthma to identify both T and non-T IL-25–responsive cells involved in pulmonary inflammation. Although previous studies have established that targeting IL-25 leads to the reduction of type 2 responses[22,23], to our knowledge this study is the first to characterize how deficiency in IL-17RB reduces the pathology of allergic asthma induced by a common environmental allergen. Other reports, including one from our laboratory, have demonstrated that eosinophils produce IL-25 (refs. 7,8), thus linking IL-25 production to eosinophilia induced by the allergic response. These data are consistent with clinical studies, as peripheral blood mononuclear cells from individuals with severe allergic rhinitis show increased IL-17RB expression[24], and polymorphisms in *IL17RB* have been associated with increased risk for severe asthma[25]. Furthermore, a recent study has identified that allergen-induced expression of IL-25 and its receptor in individuals with atopic asthma correlates with disease severity[21].

*Il17rb*$^{-/-}$ mice had reduced allergen-induced pathology, including a marked reduction in type 2 cytokines primarily associated with T2M cells, which were most prominent during persistent allergen-induced disease. The transfer of IL-25–induced T2M cells recapitulated lung pathology in IL-25–treated *Il17rb*$^{-/-}$ mice, demonstrating the sufficiency of T2M cells to mediate pathogenic responses. T2M cells seem to be steroid resistant *in vivo* and represent a distinct granulocytic population, which may have been identified in a model of pulmonary inflammation during *Nippostrongylus brasiliensis* infection[26]. T cell function was also altered in *Il17rb*$^{-/-}$ mice, demonstrating that in chronic allergic disease both T cell and non-T cell populations are contributors to type 2 cytokine–mediated pathophysiology.

Although T lymphocytes are responsible for driving allergen-specific type 2 responses, multiple reports have identified critical roles for innate immune populations. IL-25–responsive lymphoid cells have been identified in the gut in models of *N. brasiliensis* infection and have a key role in the clearance of enteric helminths[18–20]. Recent studies have identified a similar innate lymphocytic cell population in the gut, lung and nasal polyps of humans, further supporting this population's potential role in human disease[27]. In our studies, this population was present at low numbers in the lung and did not increase after allergen or IL-25 administration. Other nonlymphoid populations have also been identified as sources of type 2 cytokines that can contribute to the allergic environment, including basophils, mast cells, eosinophils and macrophages[28–38]. We did not detect substantial IL-17RB expression in any of these populations in the lungs of allergen-challenged mice. Thus, it seems that there are both Lin$^+$ and Lin$^-$ IL-25–responsive cells whose recruitment may vary depending on the mucosal surface (gut versus lung), with each cell type maintaining the capacity to produce type 2 cytokines in an antigen-independent manner.

On the basis of an analysis of T2M surface markers as well as the identification of T2M cells in the bone marrow during allergen- and IL-25–induced responses, T2M cells seem to be derived from the bone marrow. Their presence in peripheral blood of humans with asthma may represent an induced cell population that may be recruited and may accumulate in the lung during persistent or exacerbated disease. Because IL-17RB$^+$ subsets could be distinguished on the basis of their intensity of Gr-1 expression, and in light of the differing capacity for IL-4 and IL-13 production between Gr-1$^{\mathrm{mid}}$ and Gr-1$^{\mathrm{hi}}$ populations, IL-17RB$^+$Gr-1$^{\mathrm{hi}}$ cells may have functions that overlap with other CD11b$^+$Gr-1$^+$ populations, such as myeloid suppressor cells[39,40]. A recent study reported that both IL-17RA and IL-17RB can be expressed on the surface of human neutrophils[41]. Clinical studies of patients with steroid-resistant asthma demonstrated that a neutrophilic inflammatory response is predominant, and mouse studies have suggested that steroid-resistant T helper type 17 cells may explain neutrophil-mediated responses[42–50]. As IL-17RA is required for functional IL-17A and IL-25 signaling, these cytokines may share downstream signals induced following ligand binding that provide a common link for steroid resistance. The development of T2M cells probably depends on an overall type 2 immune environment and perhaps IL-25 itself.

Our data also indicate that, given the rapid accumulation of myeloid cells in lungs after IL-25 administration, there is a pool of cytokine-producing, IL-25–responsive cells capable of amplifying a type 2 response. In allergic individuals, T2M cells may have a key role in the immediate response to an environmental allergen, priming the system for a type 2 response by producing cytokines before T lymphocyte activation. T2M cells could also contribute to chronic disease, as airway epithelial damage stimulates IL-25 secretion. This concept may be especially relevant in individuals with asthma, as our studies identified high numbers of T2M-like cells in the circulation of individuals with asthma that may be recruited upon an exacerbation and accumulate in the lungs. The induction of IL-25 in airways by pathogens[51], allergens or other noxious stimuli may amplify the severity of the response by activating steroid-resistant T2M cells, especially in patients with underlying pulmonary disease. A complete understanding of the development and function of T2M cells will require further investigation; however, our findings suggest that they represent a useful biomarker and possible therapeutic target for the treatment of severe asthma.

## METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

**Accession codes.** Expression data for T2M cells, eosinophils, neutrophils and macrophages from IL-25-treated 4get mice have been deposited in the Gene Expression Omnibus with accession number GSE36392.

*Note: Supplementary information is available on the Nature Medicine website.*

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## AUTHOR CONTRIBUTIONS

B.C.P. conceived of the study, performed experiments and data analyses and wrote the manuscript. A.L.B. and M.A.S. provided intellectual contributions through technical advice and experimental design. A.P.B. coordinated recruitment of subjects with asthma. N.W.L. conceived of and supervised the study and wrote the manuscript.

## COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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# Mitochondrial transfer from bone-marrow–derived stromal cells to pulmonary alveoli protects against acute lung injury

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**Bone marrow–derived stromal cells (BMSCs) protect against acute lung injury (ALI). To determine the role of BMSC mitochondria in this protection, we airway-instilled mice first with lipopolysaccharide (LPS) and then with either mouse BMSCs (mBMSCs) or human BMSCs (hBMSCs). Live optical studies revealed that the mBMSCs formed connexin 43 (Cx43)-containing gap junctional channels (GJCs) with the alveolar epithelia in these mice, releasing mitochondria-containing microvesicles that the epithelia engulfed. The presence of BMSC-derived mitochondria in the epithelia was evident optically, as well as by the presence of human mitochondrial DNA in mouse lungs instilled with hBMSCs. The mitochondrial transfer resulted in increased alveolar ATP concentrations. LPS-induced ALI, as indicated by alveolar leukocytosis and protein leak, inhibition of surfactant secretion and high mortality, was markedly abrogated by the instillation of wild-type mBMSCs but not of mutant, GJC-incompetent mBMSCs or mBMSCs with dysfunctional mitochondria. This is the first evidence, to our knowledge, that BMSCs protect against ALI by restituting alveolar bioenergetics through Cx43-dependent alveolar attachment and mitochondrial transfer.**

Exogenous administration of purified BMSCs is protective in mouse models of organ system injury[1,2], including sepsis-induced ALI[3,4], suggesting that BMSCs might be therapeutically effective in this context[5]. However, their protective mechanisms are not entirely clear. BMSCs might improve survival in mice exposed to airway endotoxin instillation by engrafting in the alveolar epithelium and transdifferentiating to form new epithelium[6,7], although not all studies have agreed with this hypothesis[8,9], or by paracrine secretions of critical repair factors, including keratinocyte growth factor (KGF)[10], interleukin-10 (IL-10)[11,12], angiopoietin-1 (refs. 13,14), interleukin-1 receptor antagonist[15] and prostaglandin E2 (PGE2)[16].

As sepsis-induced ALI impairs lung mitochondrial bioenergetics[17–19], we considered the alternative possibility that the protective effect of BMSCs involves mitochondrial transfer to host cells. In culture, BMSCs are capable of transferring their mitochondria to endothelial cells, A549 cells and cardiomyocytes[20–22]. However, in intact lungs *in vivo*, it is not known whether airway-instilled BMSCs transfer mitochondria to lung cells and whether such a transfer is potentially therapeutic. Here we address these issues using live-lung microscopy[23,24].

## RESULTS

### mBMSCs attach to alveoli by forming Cx43-based GJCs

To establish the sepsis-induced ALI model, we airway instilled *Escherichia coli* LPS in anesthetized mice. As a control, we similarly instilled mice with PBS. We studied the lungs at 4 h and 24 h after these instillations. For the 4-h studies, we isolated and blood perfused the lungs 4 h after the LPS or PBS instillations and then immediately instilled mBMSCs intratracheally and imaged the lungs for a further 4 h[23,24]. For the 24-h studies, we gave mBMSCs by intranasal instillation in anesthetized mice 4 h after the LPS or PBS instillations and then isolated and blood perfused the lungs after 24 h for the imaging studies.

In the 4-h studies, we delineated the alveolar epithelia by loading the alveoli with a water-soluble fluorescent dye[23,24] before instilling the mBMSCs. We expressed the mitochondria-targeted fluorescent protein DsRed in the mBMSCs and confirmed that its expression was localized to the mitochondria (**Supplementary Fig. 1a**). Minutes after instilling the mBMSCs, we detected DsRed-expressing cells adjacent to the alveolar epithelium (**Fig. 1a**), confirming that the mBMSCs reached the alveoli.

In the PBS-instilled lungs (called hereafter PBS lungs), the instilled mBMSCs migrated from deep to superficial regions of the alveoli. Thus, when we viewed alveoli from these lungs in 2-μm optical sections at a fixed focal plane, we detected mBMSCs that emerged from deeper planes and then progressively occupied epithelial sites at more superficial planes at which no mBMSCs were previously present (**Fig. 1a**). Accordingly, DsRed fluorescence increased in a time-dependent manner in these lungs at the superficial sites of mBMSC emergence (**Fig. 1b**). In the 24-h studies, the PBS lungs no longer contained mBMSCs in the imaged alveoli (**Fig. 1c**) or in the lung as a whole (**Fig. 1d**), although mBMSCs were present in the adjoining subpleural interstitia (**Fig. 1c**) and in the thoracic lymph nodes (**Fig. 1e**).

Compared to the PBS lungs, in the LPS-instilled lungs (called hereafter LPS lungs), the mBMSCs migrated less (**Fig. 1b**), the subpleural interstitia contained fewer mBMSCs and the alveoli retained the mBMSCs to a greater extent (**Fig. 1c**). In addition, we detected greater

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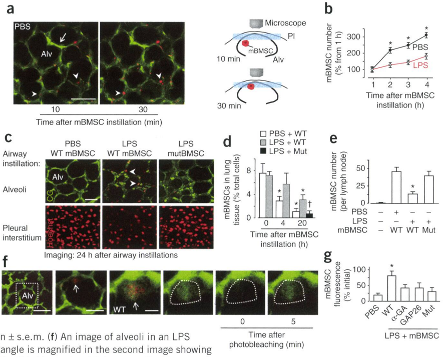

**Figure 1** mBMSCs in live alveoli. (**a**) Images of mBMSCs (arrowheads) and alveolar septa (arrow). Scale bar 30 μm. Green indicates calcein green staining, and red indicates mBMSCs with mitochondrial DsRed staining. Also shown is an illustration of mBMSC migration to the imaged plane (blue rectangle). Alv, alveolus; Pl, pleura. (**b**) Alveolar mBMSCs imaged at a fixed focal plane. Data are for 100 alveoli from six lungs in each group. *$P < 0.05$ compared to LPS by $t$ test. Data are mean ± s.e.m. (**c**) Two-photon images (optical slice thickness, 0.6 μm) at focal planes 4 μm (top) and 1 μm (bottom) deep into the visceral pleura. The experiments were replicated in six lungs for each group. Scale bar, 30 μm. MutBMSC, mBMSCs expressing mutCx43; Red indicates Hoechst 33342 staining. CG, calcein green. Arrowheads indicate sites of mBMSC retention. (**d**) Quantification of lung mBMSCs by flow cytometry. $n = 4$ lungs for each group. *$P < 0.05$ compared to 0 h, $^{\dagger}P < 0.05$ compared to the group shown on the immediate left by analysis of variance (ANOVA). Mut, mutBMSCs. Data are mean ± s.e.m. (**e**) mBMSCs in thoracic lymph nodes. $n = 6$ lymph nodes for each group. *$P < 0.05$ compared to PBS by ANOVA. Data are mean ± s.e.m. (**f**) An image of alveoli in an LPS lung (left). Scale bar, 20 μm. The area within the rectangle is magnified in the second image showing an mBMSC (arrow) in an alveolus. Scale bar, 10 μm. The third image is a higher magnification showing green fluorescence in the mBMSC (arrow). Scale bar (which applies to images 3–6), 3 μm. The fourth to sixth images are green channel renditions showing responses to photobleaching in the area within the dotted lines. Red indicates calcein red staining. (**g**) Group data showing FRAP responses in mBMSCs. $n = 15$ cells for each group. *$P < 0.05$ compared to PBS by ANOVA. Data are mean ± s.e.m. α-GA, α-glycyrrhetinic acid; GAP26, Cx43-inhibiting peptide.

numbers of retained mBMSCs in these lungs as a whole using flow cytometry (**Fig. 1d** and **Supplementary Fig. 1b**). When we airway instilled these lungs with mBMSCs containing a water-soluble dye that had a different fluorescence than that of the epithelial dye, several alveolus-attached mBMSCs spontaneously exchanged cytosolic fluorescence with the alveolar epithelium within 30 min of instillation (**Fig. 1f**).

As the dyes were membrane impermeable, this intercellular dye exchange suggested the presence of GJCs. To test this possibility, we quantified the fluorescence recovery after photobleaching (FRAP)[20], which assays GJC-dependent communication. During photobleaching, we imaged multiple optical sections in each cell's depth axis to confirm that the mBMSC fluorescence was lost throughout the cell, thereby ruling out the possibility that the fluorescence recovery was caused by intracellular dye diffusion. FRAP was present in alveolus-attached mBMSCs (**Fig. 1f**), confirming the presence of GJCs between the attached cells. Direct intra-alveolar microinfusions of the non-specific GJC blocker α-glycyrrhetinic acid or the specific Cx43-inhibiting peptide GAP26 (refs. 23,25) blocked the FRAP (**Fig. 1g**), implicating Cx43 in GJC formation.

Under culture conditions, mBMSCs that express Cx43 with a T-to-A mutation at Thr154 (mutCx43) do not form functional GJCs[20,26]. Here, mBMSCs expressing mutCx43 had markedly lower concentrations of endogenous Cx43 compared to wild-type mBMSCs (**Supplementary Fig. 1c**). These findings indicate that expression of mutant Cx43 in the mBMSCs negatively regulated endogenous Cx43 expression. When we instilled mutCx43-expressing mBMSCs in LPS lungs, they migrated out of the lungs at rates similar to those of wild-type mBMSCs in the PBS lungs (**Fig. 1d,e**). The mBMSCs expressing mutCx43 did not develop spontaneous dye exchange (data not shown) or FRAP (**Fig. 1g**), indicating that wild-type Cx43 expression in the mBMSCs was essential for GJC formation and alveolar attachment. Spontaneous dye transfer and FRAP were also absent under control conditions (instillation of PBS instead of LPS or of 3T3 cells instead of mBMSCs) (data not shown).

Within 4 h after airway instillation of LPS alone (that is, in the absence of instilled mBMSCs or other cells), Cx43 fluorescence of the alveolar epithelia increased markedly (**Fig. 2a**), indicating that LPS alone induced a Cx43-rich alveolar membrane before the alveolar arrival of mBMSCs. Line-scan fluorescence analyses in LPS lungs revealed that the distribution of Cx43 in the alveolar epithelia was spatially uneven and that the mBMSC attachment occurred at sites of high Cx43 expression (**Fig. 2b**). To determine Cx43 expression in the lungs as a whole, we instilled LPS or PBS, but no mBMSCs, in mice. Then, in primary isolates of mouse alveolar type II (AT2) cells[27] and in membrane fractions that we recovered from the lungs 4 h afterwards, mRNA analyses and immunoblots indicated that Cx43 expression was higher in LPS lungs than in PBS lungs (**Fig. 2c**). However, 24 h after instillation, the expression of Cx43 was similar in the two groups (**Fig. 2c**). In isolated AT2 cells and in an uncharacterized mixture of lung cells, mRNA analyses for known alveolar connexins[23] revealed that the mBMSCs expressed only Cx43 and that Cx43 was the only connexin that was common to both the lung cells and the mBMSCs (**Fig. 2d**). These findings implicate Cx43 as the essential connexin in the GJC formation that we detected between the mBMSCs and the alveolar epithelium. As 3T3 cells also express Cx43 (ref. 28) but did not form GJCs in the LPS lungs (as described above), we investigated the effects of cell suspension, a condition to which we exposed the mBMSCs and the 3T3 cells for ~2 h before airway instillation. Cx43 expressions were similar in plated mBMSCs and 3T3 cells. However, after we suspended the 3T3 cells for 2 h in medium, the expression of Cx43 was 44% lower in the 3T3 cells than in the mBMSCs (**Supplementary Fig. 2a**). The loss of Cx43 in suspended 3T3 cells might account for the inability of these cells to form GJCs with the alveolar epithelium *in vivo*.

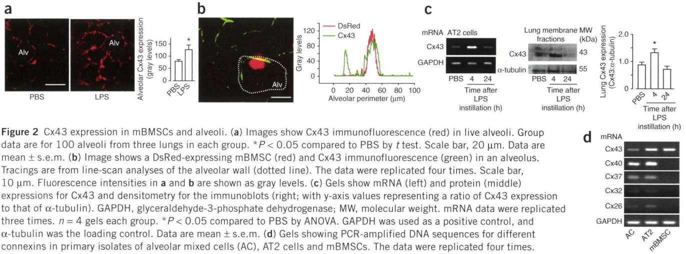

**Figure 2** Cx43 expression in mBMSCs and alveoli. (**a**) Images show Cx43 immunofluorescence (red) in live alveoli. Group data are for 100 alveoli from three lungs in each group. $*P < 0.05$ compared to PBS by *t* test. Scale bar, 20 μm. Data are mean ± s.e.m. (**b**) Image shows a DsRed-expressing mBMSC (red) and Cx43 immunofluorescence (green) in an alveolus. Tracings are from line-scan analyses of the alveolar wall (dotted line). The data were replicated four times. Scale bar, 10 μm. Fluorescence intensities in **a** and **b** are shown as gray levels. (**c**) Gels show mRNA (left) and protein (middle) expressions for Cx43 and densitometry for the immunoblots (right; with y-axis values representing a ratio of Cx43 expression to that of α-tubulin). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MW, molecular weight. mRNA data were replicated three times. $n = 4$ gels each group. $*P < 0.05$ compared to PBS by ANOVA. GAPDH was used as a positive control, and α-tubulin was the loading control. Data are mean ± s.e.m. (**d**) Gels showing PCR-amplified DNA sequences for different connexins in primary isolates of alveolar mixed cells (AC), AT2 cells and mBMSCs. The data were replicated four times.

## Alveolus-attached mBMSCs form nanotubes and microvesicles

Within 4 h after instillation of DsRed-expressing mBMSCs, LPS-treated lungs, but not PBS-treated lungs, showed that approximately half of the alveolus-attached mBMSCs had generated microvesicles and nanotubes (**Fig. 3a,b**). The released microvesicles were DsRed positive, and they moved away from the cell body at a rate (1.8 ± 0.5 μm $\text{min}^{-1}$ (mean ± s.e.m.)) similar to that of the flow of the alveolar wall liquid[24] (**Fig. 3a**), suggesting that the microvesicles contained mitochondria and were convectively transported. When plated in culture, wild-type mBMSCs displayed similar behavior in that they spontaneously formed mitochondria-transporting nanotubes (**Supplementary Fig. 2b**). These plated wild-type mBMSCs intercommunicated induced increases in the fluorescence of a dye that reports the cytosolic $Ca^{2+}$ concentration, an effect that was not present in mBMSCs expressing mutCx43 (**Supplementary Fig. 2c**), suggesting that the $Ca^{2+}$ communication was GJC dependent. Although mutCx43-expressing mBMSCs also formed nanotubes in culture (data not shown), they did not support $Ca^{2+}$ communication (**Supplementary Fig. 2c**). Moreover, they did not form nanotubes or microvesicles when they were airway instilled in LPS lungs (**Fig. 3b**), presumably because they did not attach to alveoli (**Fig. 1d,g**). Nanotubes and microvesicles also did not form in wild-type mBMSCs that we instilled in PBS lungs or in 3T3 cells that we instilled in LPS lungs (data not shown). Microvesicle budding was not present in a subset of wild-type mBMSCs that did not form GJCs in LPS lungs, indicating that mBMSC attachment to the alveolar epithelium was essential for this budding to occur. Flow cytometry determinations in the supernatants of LPS lungs indicated that mBMSC microvesicle formation was extensive in these lungs as a whole

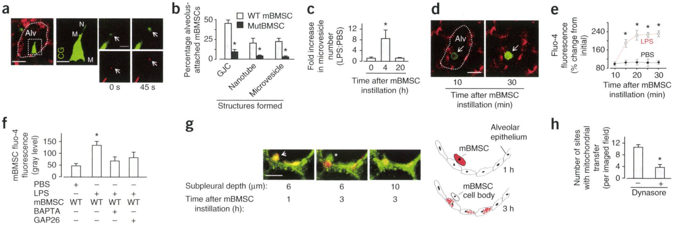

**Figure 3** Responses of alveolus-attached mBMSCs 4–8 h after LPS instillation. (**a**) Image of an LPS lung (left) showing an mBMSC (green) in an alveolus (red). The alveolar margins are marked with a dotted line. Scale bar, 10 μm. The second image is a higher magnification of the selected region (indicated by a rectangle in the left image) showing the mBMSC in green rendition. M, microvesicles; N, nanotube. Scale bar, 5 μm. The four images on the right show a microvesicle (arrows) at different time points in green (calcein green) and red (DsRed) renditions. Scale bar, 3 μm. (**b**) Group data for LPS lungs. $n = 40$ mBMSCs from five lungs in each group. $*P < 0.05$ compared to the corresponding wild-type mBMSC group by *t* test. Data are mean ± s.e.m. (**c**) The number of microvesicles in LPS lungs versus those in PBS lungs as determined by flow cytometry of supernatants derived from homogenates of lungs isolated at the indicated time points after mBMSC instillation. $n = 4$ lungs in each group. $*P < 0.05$ compared to 0 h by ANOVA. Data are mean ± s.e.m. (**d**) Images of an mBMSC (arrows) stained with a $Ca^{2+}$ dye, fluo-4, in a live alveolus (red). The alveolar margin is marked by the dotted line (left image). Scale bar, 10 μm. (**e**,**f**) Group data showing a time course of fluo-4 responses in mBMSCs (**e**) and the effects of the indicated treatments (**f**). $n = 9$ mBMSCs from three lungs in each group. $*P < 0.05$ compared to PBS by ANOVA. Data are mean ± s.e.m. WT, wild type; BAPTA, $Ca^{2+}$ chelator. (**g**) Images showing an mBMSC (arrow) located adjacent to the alveolar epithelium (green). The illustration on the right depicts the imaging data at two time points. Mitochondria are shown in red, and the mBMSC cell body is marked by an asterisk (middle image). Scale bar, 5 μm. (**h**) Group data for epithelial engulfment. $n = 7$ imaged fields from three lungs in each group. $*P < 0.05$ compared to the dynasore-negative group by *t* test. Data are mean ± s.e.m.

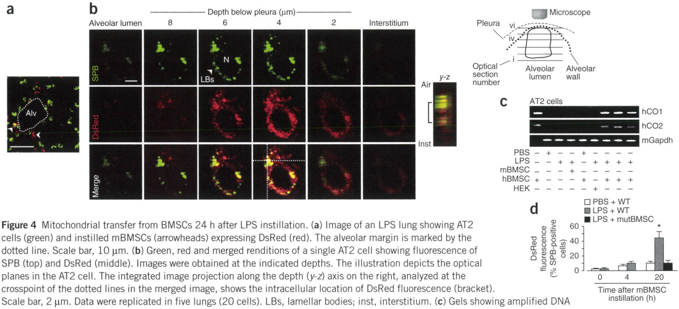

**Figure 4** Mitochondrial transfer from BMSCs 24 h after LPS instillation. (**a**) Image of an LPS lung showing AT2 cells (green) and instilled mBMSCs (arrowheads) expressing DsRed (red). The alveolar margin is marked by the dotted line. Scale bar, 10 μm. (**b**) Green, red and merged renditions of a single AT2 cell showing fluorescence of SPB (top) and DsRed (middle). Images were obtained at the indicated depths. The illustration depicts the optical planes in the AT2 cell. The integrated image projection along the depth (*y-z*) axis on the right, analyzed at the crosspoint of the dotted lines in the merged image, shows the intracellular location of DsRed fluorescence (bracket). Scale bar, 2 μm. Data were replicated in five lungs (20 cells). LBs, lamellar bodies; inst, interstitium. (**c**) Gels showing amplified DNA sequences from isolated AT2 cells. HEK, human embryonic kidney cells; mGapdh, mouse Gapdh; hCO1, human CO1; hCO2, human CO2. Data were repeated four times. (**d**) Group data from flow cytometry determinations. *n* = 4 lungs for each group. $^*P < 0.05$ compared to 0 h by ANOVA. Data are mean ± s.e.m.

(**Fig. 3c**), increasing in the 4 h after mBMSC instillation but decreasing at 20 h after instillation (**Fig. 3c** and **Supplementary Fig. 2d**).

To gain mechanistic insights into nanotube formation[22,29], we airway instilled wild-type mBMSCs containing a cytosolic $Ca^{2+}$ indicator in LPS and PBS lungs. In the LPS lungs, but not the PBS lungs, alveolar attachment caused time-dependent increases in $Ca^{2+}$ concentration in the mBMSCs (**Fig. 3d–f**). As alveolar treatment with GAP26 blocked this effect (**Fig. 3f**), it seems that the $Ca^{2+}$ increase was GJC dependent. When we instilled LPS lungs with mBMSCs that we previously loaded with a $Ca^{2+}$ chelator, the mBMSCs successfully attached to the alveolar wall but did not increase their $Ca^{2+}$ concentrations or form nanotubes and microvesicles (**Supplementary Fig. 2d**), suggesting that these structural responses resulted from gap junctional $Ca^{2+}$ communication.

## mBMSCs transfer mitochondria to the alveolar epithelium

In the LPS lungs from the 4-h studies, mitochondrial transfer was evident by the loss of DsRed fluorescence in alveolus-attached mBMSCs and by the gain of this fluorescence by the adjoining epithelium. We did not see mitochondrial transfer in the PBS lungs in the 4-h studies. When we imaged the alveolar epithelia of LPS lungs at different optical sections, the epithelial internalization of the mBMSC mitochondria was evident in the merging of the colors from DsRed and calcein, which resulted in intermediate pseudocolors in the epithelial cytosol (**Fig. 3g**). Trypan Blue, a cell-impermeable fluorescence quencher that decreases extracellular fluorescence[30], did not decrease the DsRed fluorescence (data not shown), confirming that the DsRed fluorescence was internalized. After the mitochondrial transfer, mBMSC cell bodies lacking DsRed-labeled mitochondria remained attached to the apical alveolar epithelia (**Fig. 3g**) but did not intercalate in the alveolar wall.

The possibility that mBMSC microvesicles were engulfed by the epithelium was indicated by the fact that the dynamin inhibitor[31] dynasore blocked the mitochondrial transfer (**Fig. 3h**), implicating endocytosis in the engulfment mechanism. In support of this possibility, DsRed-positive microvesicles, which we recovered from LPS lungs in which we previously instilled wild-type mBMSCs, stained positively for Cx43 (**Supplementary Fig. 2e**). Further, instillation of mutCx43-expressing mBMSCs instead of wild-type mBMSCs led to markedly lower recovery of microvesicles from LPS lungs (**Fig. 3b**).

Internalization of DsRed-expressing mBMSC mitochondria was most clearly evident in AT2 cells 24 h after the instillation of LPS and mitochondrial-DsRed–expressing mBMSCs. To determine the internalization in AT2 cells, we loaded alveoli with fluorescent surfactant protein B (SPB) that localizes to lamellar bodies that are present only in AT2 cells (**Fig. 4a**). Alveolar type 1 (AT1) cells, comprising the alveolar segment that lacked SPB fluorescence, did not show evidence for DsRed internalization. We imaged single AT2 cells by confocal microscopy in a series of 1-μm optical sections along the depth axis from the plane of the alveolar lumen toward the pleural plane. During this serial imaging, the images transitioned from dark signals at the air-filled plane to fluorescent signals at the cytosolic planes of the AT2 cell. Also evident were mBMSC mitochondria as DsRed-containing particles that were interspersed with perinuclear lamellar body clusters (**Fig. 4b**), affirming the intracellular localization of the acquired mitochondria in AT2 cells.

To further validate the mitochondrial transfer, we prepared LPS or PBS lungs as described above, except that we instilled hBMSCs instead of mBMSCs. Twenty-four hours after these instillations, we isolated the AT2 cells and a mixed population of lung cells (**Supplementary Fig. 3a–f**) in which we PCR amplified DNA sequences coding for human cytochrome oxidases 1 and 2 (*CO1* and *CO2*) (**Fig. 4c** and **Supplementary Fig. 3g**), which are transcribed by mitochondrial DNA. The presence of these human DNA sequences in mouse AT2 cells derived from LPS lungs but not PBS lungs further affirmed that after LPS-induced lung injury, AT2 cells acquired mitochondria from the instilled hBMSCs. In support of this, we carried out flow cytometry studies on primary isolates of lung cells from LPS and PBS lungs. In LPS lungs, DsRed- and SPB-positive cells localized in the same cell cluster, which is consistent with the interpretation that the mBMSC mitochondria were located in the AT2 cells (**Fig. 4d** and **Supplementary Fig. 3h,i**). No mitochondrial transfer was evident in

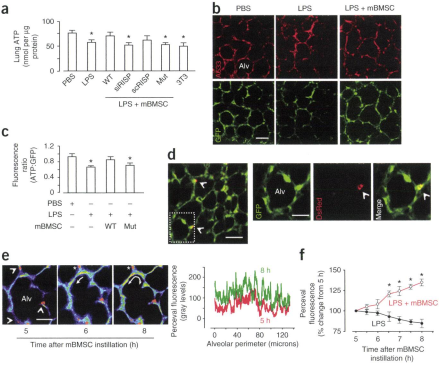

**Figure 5** Effect of mBMSCs on alveolar bioenergetics. (**a**) Group data from colorimetric determinations in lung homogenates after instillation with PBS or LPS alone or with LPS followed by either wild-type (WT), mutCx43-expressing (Mut), siRISP-treated or scRISP-treated mBMSCs. 3T3 cells were also used as a further control. $n = 4$ lungs for each group. *$P < 0.05$ compared to PBS by ANOVA. Data are mean ± s.e.m. (**b**) Images showing immunofluorescence (top) and GFP fluorescence (bottom) of the ATP probe Perceval. A633, Alexa fluor 633. Scale bar, 20 μm. (**c**) Group data for ATP fluorescence. $n = 3$ imaged fields for each group. *$P < 0.05$ compared to PBS by ANOVA. Data are mean ± s.e.m. (**d**) Image (left) obtained 24 h after LPS instillation showing the alveolar fluorescence of Perceval (green) and mBMSC mitochondria (red and arrowheads). Scale bar, 20 μm. To the right are magnified images of the selected region (indicated by the rectangle in the left image) showing an alveolus in the green, red and merge renditions. DsRed fluorescence is indicated (arrowheads). Scale bar, 10 μm. (**e**) The yellow pseudocolor in the marked regions (asterisk) shows changes in alveolar GFP fluorescence (curved arrows). We superimposed the red channel rendition of DsRed mitochondria on the alveolar image (arrowheads in left image). The tracings are from line-scan analyses. Scale bar, 10 μm. (**f**) Group data showing changes in Perceval fluorescence. $n = 5$ areas in three lungs for each group. *$P < 0.05$ compared to LPS alone by ANOVA. Data are mean ± s.e.m.

PBS lungs after instillation of wild-type mBMSCs or in LPS lungs after instillation of mutCx43-expressing mBMSCs (**Fig. 4d**).

## mBMSC mitochondria increase alveolar ATP

We confirmed that the concentration of ATP was lower in the LPS lungs than in the PBS lungs[32,33]. We showed further that after we instilled wild-type mBMSCs, the concentrations of ATP were similar in the two groups (**Fig. 5a**). Major ATP production in the lung occurs through mitochondrial electron transport, which is inhibited by siRNA knockdown of the Rieske iron-sulfur protein (RISP) of complex III[30,34]. In cultured mBMSCs expressing anti-RISP siRNA (siRISP), but not scrambled siRNA (scRISP), ATP concentrations were less than in wild-type mBMSCs (**Supplementary Fig. 4a**,**b**). These findings affirm the role of RISP in ATP production in mBMSCs.

Expression of siRISP did not adversely affect the mBMSCs in culture in that the BMSCs expressing siRISP showed no evidence of apoptosis (**Supplementary Fig. 4c**) and their mitochondrial protein content was not different from that of wild-type mBMSCs (data not shown). The siRISP-expressing mBMSCs were functionally competent, forming alveolar attachments (data not shown) and transferring mitochondria to AT2 cells (**Supplementary Fig. 3i**). However, neither siRISP-expressing nor mutCx43-expressing mBMSCs could rescue ATP production in LPS lungs (**Fig. 5a**).

The availability of the fluorescent ATP probe GlnKII-GFP (Perceval)[35] enabled direct determinations of single-cell ATP concentrations in live alveoli. We confirmed expression of the probe in LPS and PBS lungs by immunoblotting for GFP in the lung tissue (**Supplementary Fig. 5a**). Both immunofluorescence and GFP fluorescence of the probe were widely expressed in the alveolar

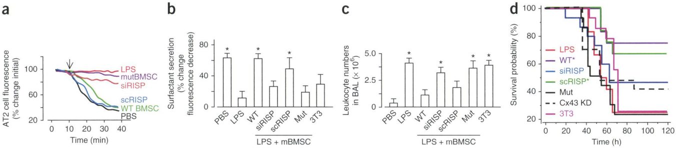

**Figure 6** Effect of mBMSCs on injury outcomes. (**a**,**b**) Inflation-induced (arrow) surfactant secretion for single experiments (**a**) and for each group as a whole (**b**). $n = 12$ cells in four lungs for each group. *$P < 0.05$ compared to LPS alone by ANOVA. Data are mean ± s.e.m. (**c**) Group data for leukocyte numbers in BAL. $n = 4$ lungs for each group. *$P < 0.05$ compared to PBS by ANOVA. Data are mean ± s.e.m. (**d**) Plots for mouse survival after instillation with LPS followed by mBMSC instillation. Cx43 KD, mBMSCs treated with siRNA against Cx43. $n = 15$ mice for each group., *$P < 0.05$ compared to LPS by Kaplan-Meier test.

epithelia in all regions of the lung's surface (**Fig. 5b**). We confirmed that the expressed ATP fluorescence was appropriately responsive to cell ATP concentrations, which we altered through glucose depletion and replenishment in the lung perfusate (**Supplementary Fig. 5b**)[35].

In the 24-h experiments, alveolar ATP fluorescence was lower in LPS lungs than in PBS lungs (**Fig. 5b**). However, in both groups of lungs, the fluorescence was similar after we instilled wild-type, but not mutCx43-expressing, mBMSCs (**Fig. 5b,c**). In the LPS lungs, mitochondrial transfer to a localized alveolar segment increased the ATP fluorescence not only throughout the same alveolus but also in 8–10 of the adjacent alveoli (**Fig. 5d**). Within 5–8 h of instilling the mBMSCs in both groups, time-lapse imaging indicated that the increase of ATP fluorescence spread progressively from the site of mitochondrial transfer to other sites in the alveolar wall (**Fig. 5e**). In the absence of mBMSC instillation, time-lapse ATP fluorescence tended to decrease, although this change was not statistically significant (**Fig. 5f**).

## mBMSC mitochondria protect against ALI

To assay an ATP-dependent alveolar functional response, we quantified surfactant secretion of intact alveoli from AT2 cells as the rate of lamellar body exocytosis that was responsive to a brief lung expansion[36]. LPS lungs, but not PBS lungs, lacked this secretory response. However, we could induce the secretion in LPS lungs by instilling wild-type mBMSCs (**Fig. 6a,b**) but not mBMSCs expressing siRISP or mutCx43 (**Fig. 6a,b**).

Globally, LPS induced the responses expected to result from ALI, namely increased leukocytosis in the bronchoalveolar lavage (BAL) (**Fig. 6c**), increased albumin leakage in the BAL (**Supplementary Fig. 6**) and decreased mouse survival time (**Fig. 6d**). In confirmation of previous reports[11,16], wild-type mBMSCs markedly abrogated these responses. Our new findings are that the instillation of mBMSCs expressing siRISP, siRNA against Cx43 or mutCx43 did not ameliorate the effects of LPS on BAL leukocytosis or mouse survival (**Fig. 6c,d**).

## DISCUSSION

Our findings indicate that alveolar attachment and, hence, lung retention of the migration-prone BMSCs, is crucial for the ensuing protective effects against ALI. After alveolar attachment in LPS lungs, BMSCs transferred their mitochondria to the alveolar epithelium, an event that occurred progressively for 24 h, accounting for their protective effect. BMSCs carrying defective mitochondria did not confer such protection. Cx43 was the crucial factor underlying the alveolar attachment of wild-type BMSCs that generated Cx43-expressing nanotubes and microvesicles in a $Ca^{2+}$-dependent manner in LPS lungs. Epithelial engulfment of the released microvesicles might have contributed to the mitochondrial transfer. Microvesicles formed extensively in the LPS lungs, and their parenchymal dispersal might have been convective[24,37]. Together, these findings provide the first *in vivo* evidence, to our knowledge, that BMSCs establish epithelial interactions in LPS lungs, form microstructures and transfer their mitochondria to host cells.

This mitochondrial transfer increased alveolar ATP concentrations in LPS lungs in a Cx43-dependent manner. Single-cell ATP determinations, the first in an intact organ, to our knowledge, indicated that the increase in ATP concentration occurred at the transfer site as well as in the adjoining alveoli, affirming that the transferred mitochondria were live and capable of ATP generation in the recipient epithelium. Inflation-induced alveolar surfactant secretion, a major ATP-dependent process that regulates alveolar stability[38,39], provides a functional metric of alveolar bioenergetics. That we could induce this secretion in LPS lungs only after instillation of wild-type BMSCs, but not of BMSCs carrying dysfunctional mitochondria, underlines the key role of bioenergetics restoration in cell repair therapy.

We confirmed that the instillation of mBMSCs increased mouse survival in LPS-induced ALI[11], and we showed further that this survival advantage was lost if the mBMSCs contained dysfunctional mitochondria or GJC-incompetent Cx43 or if they were depleted of Cx43. These findings further support Cx43-dependent mechanisms and the transfer of viable mitochondria in the protective response of mBMSCs. Although long-term consequences of mitochondrial transfer are currently not understood, the transfer was evidently sufficiently rapid to be protective in the acute phase of lung injury.

An unresolved issue is the relative role of paracrine secretion[11,12,16] compared to that of mitochondrial transfer in the BMSC protective effect. Although mitochondrial loss in alveolus-attached BMSCs could decrease ATP concentrations in these cells, thereby decreasing their secretory capacity, protective paracrine secretions could continue from nonattached, mitochondria-competent BMSCs. The combined effects of these different sets of BMSCs require further clarification. Also unclear is how an increase of alveolar ATP concentrations repairs the alveolar epithelial and endothelial barriers that are pathologically defective in ALI. Although these issues require attention, we propose that exogenous BMSCs provide a 'Trojan horse' therapeutic strategy for supplying fresh mitochondria to injured cells, thereby enhancing cellular bioenergetics and improving organ function in ALI and other inflammatory diseases.

## METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

*Note: Supplementary information is available on the Nature Medicine website.*

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AUTHOR CONTRIBUTIONS

M.N.I. carried out the experiments, prepared the figures and wrote the initial manuscript. S.R.D. carried out the protein gel assays. M.T.E. contributed to the flow cytometry experiments. M.W. carried out the RNA isolation and RT-PCR experiments. L.S. and S.K.Q. carried out the plasmid amplifications and BMSC transfections. K.W. contributed to the epithelial cell isolation experiments. D.J.R. contributed to the imaging experiments. S.B. contributed to the experimental design. J.B. was responsible for the overall project and wrote the initial manuscript. All authors edited the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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# *RBM20*, a gene for hereditary cardiomyopathy, regulates titin splicing

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**Alternative splicing has a major role in cardiac adaptive responses, as exemplified by the isoform switch of the sarcomeric protein titin, which adjusts ventricular filling. By positional cloning using a previously characterized rat strain with altered titin mRNA splicing, we identified a loss-of-function mutation in the gene encoding RNA binding motif protein 20 (*Rbm20*) as the underlying cause of pathological titin isoform expression. The phenotype of Rbm20-deficient rats resembled the pathology seen in individuals with dilated cardiomyopathy caused by *RBM20* mutations. Deep sequencing of the human and rat cardiac transcriptome revealed an RBM20-dependent regulation of alternative splicing. In addition to titin (*TTN*), we identified a set of 30 genes with conserved splicing regulation between humans and rats. This network is enriched for genes that have previously been linked to cardiomyopathy, ion homeostasis and sarcomere biology. Our studies emphasize the key role of post-transcriptional regulation in cardiac function and provide mechanistic insights into the pathogenesis of human heart failure.**

RNA splicing, involving the spliceosome and the dynamic interaction of RNA and protein complexes[1], is tightly regulated by proteins that repress or activate splice site selection. Among these are serine/arginine-domain–containing (SR) proteins and SR-related factors, which modulate the binding and assembly of the spliceosome complex and thereby determine the differential inclusion of exons in the mature transcript[2]. Few mutations in spliceosome components have been described, suggesting that the defects resulting from these mutations are incompatible with life[3], and only a relatively small number of human diseases have been attributed to the *trans* effects of mutations in RNA binding proteins, including cancer, neurological diseases and muscular atrophy[4]. Nevertheless, mutations affecting splice consensus sites (*cis* effects) cause a wide range of disorders, including cardiac disease[5].

Changes in gene and isoform expression are relevant to both adaptive effects of cardiac physiology and to cardiac disease—among them alternative splicing of the mRNA encoding the giant protein titin. Titin is a sarcomeric protein that determines the structure and biomechanical properties of striated muscle, and it has been associated with human cardiac disease[6]. Both alternative splicing[7–10] and post-translational modification tune titin-based passive tension, a key contributor to efficient ventricular filling[11,12]. Titin phosphorylation in response to adrenergic stimulation has been attributed to the action of protein kinase A on the elastic N2B region[11]. Despite established links between the alternative splicing of titin and both thyroid hormone signaling and insulin signaling[13–15], the splice factors that determine titin isoform transitions are unknown.

Here we studied a spontaneously occurring rat strain deficient in titin splicing that results in persistent expression of a giant titin isoform (N2BA-G) in adult cardiac tissue[16]. We used genome-wide mapping approaches to identify mutation of *Rbm20* as the underlying cause for dysregulated titin splicing. Notably, human RBM20 has recently been linked to human dilated cardiomyopathy (DCM) and has been speculated to affect splicing[17,18]. However, the function, regulation and substrates of RBM20 are unknown. We show that Rbm20 deficiency in rats leads to many phenotypic features that are observed in individuals with cardiomyopathy related to mutant *RBM20*, suggesting conserved RBM20 function. We then studied the function of RBM20 as a global regulator of cardiac alternative splicing and document considerable overlap of post-transcriptionally regulated genes that depend on RBM20. Our studies not only offer mechanistic insights but also provide functional annotation of RBM20 substrates that contribute to cardiomyopathy and heart failure, a major source of mortality in the developed world.

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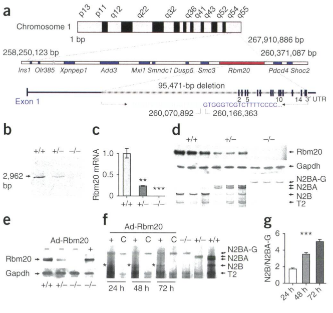

**Figure 1** Mapping of the titin splice defect and validation of *RBM20* as the affected gene. (**a**) As depicted in schematic diagrams, in a titin splice-deficient rat strain, the quantitative trait locus resided in a 2.1-Mb interval on the long arm of chromosome 1 (1q55). The coding sequences of all known genes in the interval were unchanged, with the exception of the *Rbm20* gene, which contained a 95 kb-deletion that eliminated all exons following exon 1. The endpoints of the deletion are shown in the 5′ and 3′ breakpoint (light and dark blue, respectively). UTR, untranslated region. (**b**) Southern blot of genomic DNA confirming the deletion with the loss of a 3-kb internal HindIII fragment in rats homozygous for the *Rbm20* deletion (–/–) and a reduced amount of this fragment in rats heterozygous for the deletion (+/–) compared to wild-type (+/+) rats. (**c**) Rbm20 mRNA levels normalized to 18S ribosomal RNA and wild-type RNA levels, with a 76% reduction of Rbm20 RNA expression in heterozygous rats and no (<1%) Rbm20 RNA expression in homozygous rats. $n = 3$ rats per group. $^{**}P < 0.01$, $^{***}P < 0.001$ (one-way analysis of variance (ANOVA)). Data are means ± s.e.m. (**d**) N2B titin isoforms in the indicated groups of rats, as determined by agarose gel electrophoresis. T2 refers to a proteolytic fragment of titin. Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was used as a loading control. (**e**) Rbm20 expression in rat neonatal cardiomyocytes of the indicated genotypes after infection with Rbm20-expressing (+) or control (–) adenovirus. Ad-Rbm20, Rbm20-expressing adenovirus. (**f**) N2B titin isoforms in neonatal RBM20-deficient cardiomyocytes with Rbm20 (+) or control (C) adenovirus at the indicated time after adenoviral infection. Untransfected neonatal cardiomyocytes of the indicated genotypes were also analyzed. The asterisks indicate upregulation of the N2B isoform after Ad-Rbm20 infection. (**g**) Quantification of titin isoform expression as indicated in **f**, based on three rats per group. $^{***}P < 0.001$ by one-way ANOVA. Data are means ± s.e.m.

## RESULTS

### Identification of Rbm20 as an effector of titin isoforms

We used a backcross between rats deficient in titin splicing[16,19] and the Brown Norway reference rat and identified a ~2-Mb interval on chromosome 1q55 that contains nine genes (**Fig. 1a** and **Supplementary Fig. 1**). Sequence analysis of the coding regions of these genes showed that only the *Rbm20* gene differed between rats with and without the splicing deficiency: a 95-kb deletion removing exons 2–14 was associated with the splicing deficiency. These exons encode the RNA binding motif domain, arginine/serine-rich (RS) domain and $Zn^{2+}$ finger domain of the protein. We confirmed deletion of these exons by Southern blot, quantitative RT-PCR (qRT-PCR), and western blot analyses in rats heterozygous or homozygous for the *Rbm20* deletion (**Fig. 1b–d**). In addition, we genotyped the *Rbm20* deletion in all 191 rats from two independent backcrosses and found complete cosegregation between the titin splice defect and the presence of the *Rbm20* deletion (**Supplementary Fig. 1a**).

We then used adenoviral gene delivery to re-express Rbm20 in Rbm20-deficient rat cardiomyocytes and reconstituted expression of the short titin isoform (**Fig. 1e–g**). The combination of rodent genetics and the reversion of the splice-deficient phenotype establishes Rbm20 as a *trans*-acting factor regulating titin isoform expression.

### RBM20 regulates alternative splicing in striated muscle

We next investigated the function of RBM20 in alternative splicing. In the nuclei of mouse atrial myocyte HL-1 cells, Rbm20 partially colocalized with the splice factors Ptbp1 (polypyrimidine tract binding protein 1) and U2AF65 (U2 small nuclear RNA auxiliary factor 65-kDa subunit) (**Fig. 2a**). The latter protein contains an RS domain that mediates its recruitment to sites of active splicing[20]. Using crosslinking and immunoprecipitation experiments, we found that Rbm20 binds total RNA (**Fig. 2b**). Moreover, we confirmed the specific activity of Rbm20 on titin RNA using a splice reporter assay in which exclusion of the titin exon PEVK8, which contributes to titin's elasticity, results in reduced reporter activity (**Fig. 2c**). In both mouse myoblasts (C2C12 cells) and human fibroblasts (HEK293 cells), expression of RBM20 led to the exclusion of the PEVK exon 8, which contains the firefly luciferase reporter (**Fig. 2d** and **Supplementary Fig. 2**), and the assay was validated at the RNA level (**Supplementary Fig. 2**). Thus, Rbm20 is sufficient to drive the alternative splicing of titin's PEVK region in muscle and non-muscle cells, and this splicing evidently does not rely on a cardiac-specific cofactor. Expression of either of the splicing factors PTBP1 or HuD (ELAV-like 4)[21,22] did not result in this splicing event, excluding nonspecific effects of RNA binding proteins (**Fig. 2e**). Rbm20 promoted splicing in a dose-dependent fashion (**Fig. 2f**).

Rbm20 is predominantly expressed in striated muscle, with highest expression in the heart (**Fig. 2g**). In differentiating myoblasts, Rbm20 expression correlated with sarcomere assembly (**Fig. 2h**): expression peaked when α-actinin was localized mainly in mature Z bodies within the nascent myofiber (punctuate α-actinin staining) and expression declined as the sarcomeres continued to mature (**Fig. 2i**). Experimental cardiomyopathies in mice—both genetic and viral—did not affect Rbm20 expression (**Supplementary Fig. 3**).

### Rats and humans share RBM20-dependent pathology

Adult rats heterozygous or homozygous for the *Rbm20*-null allele had left ventricular dilatation as determined by echocardiography. Although left ventricular diastolic diameters were significantly larger in these rats compared to wild-type rats, there were no differences in the systolic ventricular dimensions or contractility indices, including fractional shortening, between any of the groups (**Fig. 3a,b**).

In *Rbm20*-null heterozygous or homozygous rats, subendocardial fibrosis increased with age and was accompanied by electrical abnormalities, such as widening of the QRS complex, atrioventricular conduction delay and a predisposition to arrhythmia (**Fig. 3c–f** and **Supplementary Figs. 4–6**). Rbm20-deficient rats experienced sudden death (**Fig. 3f**), similar to humans with RBM20 mutations who can experience sudden death well after adolescence[17,18]. Thus, Rbm20-deficient rats mirror the pathological features of RBM20-deficient humans[17,18] (**Supplementary Table 1**).

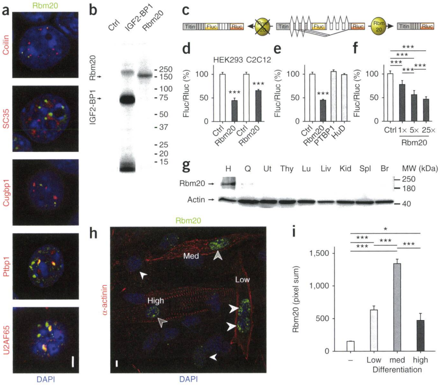

**Figure 2** Analysis of Rbm20 expression and function. (**a**) In the nuclei of HL-1 cells, Rbm20 partially colocalized with the splice factors U2AF65 and Ptbp1 but not with the other splice factors tested (Cugbp1 and SC35) or with coilin, a marker for Cajal bodies. Scale bar, 5 μm. (**b**) Binding of protein to total RNA, as detected by immunoprecipitation from lysates of cells with 5′ $^{32}$P labeling of total RNA, followed by autoradiography. Lysates were from untransfected HEK293 cells (Ctrl), HEK293 cells overexpressing Rbm20, or HEK293 cells overexpressing IGF2-BP1 (a positive control). (**c**) Specific activity of Rbm20 on titin mRNA monitored in a cell-based splice-reporter assay using the exons that encode the most N-terminal titin PEVK region (5′ PEVK exons) and firefly and *Renilla* luciferase reporters (Fluc and Rluc, respectively). Expression of Rbm20 leads to the exclusion of the Fluc-containing exon. Black lines connecting exons indicate exon junctions. The ratio of Fluc to Rluc activity reflects splice activity. (**d**) The ratio of Fluc to Rluc in empty vector control–transfected or Rbm20-expressing HEK293 cells and C2C12 myoblasts (two independent experiments). (**e**) The ratio of Fluc to Rluc in empty vector control–transfected, Rbm20-, PTBP1- or HuD-transfected HEK293 cells (three independent experiments). (**f**) The ratio of Fluc to Rluc in HEK293 cells transfected with increasing amounts of Rbm20 plasmid (1×, 5× or 25× the initial amount of Rbm20 reproduced in two independent experiments). (**g**) Rbm20 protein expression in the indicated tissues of rats at 2 months of age. H, heart; Q, quadriceps; Ut, uterus; Thy, thymus; Lu, lung; Liv, liver; Kid, kidney; Spl, spleen; Br, brain; MW, molecular weight. Actin was used as a loading control. (**h**) C2C12 cells stained for α-actinin (red) as a marker of sarcomere maturation and staged for low, medium and high differentiation based on the fluorescence pattern. Rbm20 expression (green) was restricted to nuclei (DAPI, blue). Scale bar, 5 μm. Arrowheads point to nuclei. (**i**) Quantification of Rbm20 expression in undifferentiated C2C12 cells (–) and in cells at low, medium and high states of differentiation, as shown in **h**. The pixel sum is a measure of the combined signal intesity over the nuclear area. $^{*}P = 0.05$, $^{***}P < 0.001$. One-way ANOVA; $n > 15$ cells per group. All data are means ± s.e.m.

## Titin splicing in a patient with a novel *RBM20* mutation

To validate RBM20-dependent titin splicing and its relevance in human disease, we investigated an individual with a heterozygous mutation in *RBM20* resulting in a S635A amino acid substitution. The affected amino acid residue resides in an RS domain within an evolutionary highly conserved region of the protein (**Fig. 4a,b**). This 34-year-old subject suffered from severe cardiomyopathy and had a clinical presentation that matched recently published patient data[17,18]. We tested

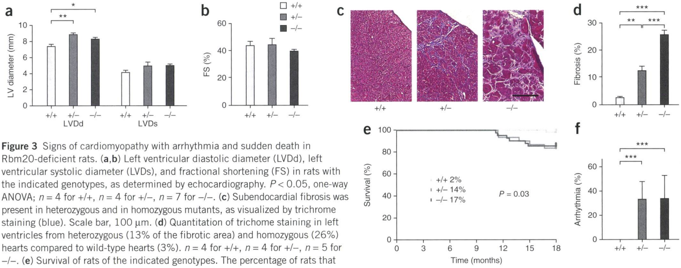

**Figure 3** Signs of cardiomyopathy with arrhythmia and sudden death in Rbm20-deficient rats. (**a**,**b**) Left ventricular diastolic diameter (LVDd), left ventricular systolic diameter (LVDs), and fractional shortening (FS) in rats with the indicated genotypes, as determined by echocardiography. $P < 0.05$, one-way ANOVA; $n = 4$ for +/+, $n = 4$ for +/–, $n = 7$ for –/–. (**c**) Subendocardial fibrosis was present in heterozygous and in homozygous mutants, as visualized by trichrome staining (blue). Scale bar, 100 μm. (**d**) Quantitation of trichome staining in left ventricles from heterozygous (13% of the fibrotic area) and homozygous (26%) hearts compared to wild-type hearts (3%). $n = 4$ for +/+, $n = 4$ for +/–, $n = 5$ for –/–. (**e**) Survival of rats of the indicated genotypes. The percentage of rats that had died by 18 months of age is indicated. $n = 56$ for +/+, $n = 30$ for +/–, $n = 41$ for –/–. $P = 0.03$ by log-rank (Mantel-Cox) test indicates a statistical difference among the three groups analyzed. (**f**) Percentage of rats that could be induced to have arrhythmia per genotype; $n = 4$ for +/+, $n = 5$ for +/–, $n = 3$ for –/–. All data are means ± s.e.m. $^{*}P = 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

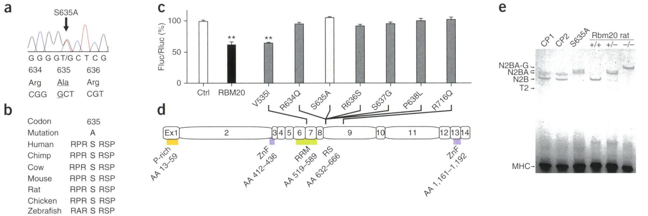

**Figure 4** Identification of a novel and functionally relevant RBM20 mutation in an individual with severe cardiomyopathy and arrhythmia. (**a**) A sequence trace showing the T to G substitution that results in an S635A change (underlined) within the RS domain. (**b**) Alignment of the region affected by the mutation in the indicated species. (**c**,**d**) The ratio of Fluc to Rluc in the reporter splicing assay for untransfected cells (Ctrl) and cells transfected with wild-type RBM20 or the indicated RBM20 mutants. The schematic diagram of RBM20 in **d** indicates the location of each of the mutations in RBM20. $n = 8$ for each group. Data are mean ± s.e.m. $^{**}P < 0.01$, one-way ANOVA. AA, amino acid; Ex, exon; P-rich, proline rich; RRM, RNA recognition motif; ZnF, zinc finger. (**e**) Titin isoform expression as determined by SDS–agarose gel electrophoresis. Samples were obtained from the human subject with DCM with S635A-mutant RBM20, control cardiomyopathy subjects of unrelated cause (CP1 and CP2) and rats of the indicated genotypes. MHC, myosin heavy chain

3,000 alleles from individuals without cardiomyopathy (who were ethnically matched to the affected individual) as controls and were unable to detect this variant; in addition, the S635A variant has not been reported in the dbSNP database or by the 1000 Genomes Project, indicating that it is not a frequent polymorphism. We used the splice reporter assay to test whether the newly identified S635A variant and all other known human RBM20 variants[17,18] have functional effects. All of the variants analyzed showed a complete loss of function with the exception of the conserved amino acid change V535I, which is caused by the only identified mutation not within exon 9 (**Fig. 4c,d**).

We next tested cardiac biopsy material from the individual with the S635A variant, as well as cardiac tissue from two subjects with

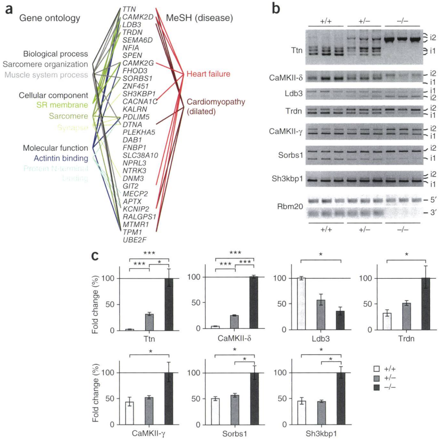

**Figure 5** Characterization of RBM20-dependent isoform expression. (**a**) A conserved set of 31 genes with RBM20-dependent alternative splicing identified by next-generation sequencing of humans and rats, including *TTN* as the most differentially spliced gene. Gene ontology terms provide a unified assignment of gene attributes that does not include disease relation. The latter is provided through MeSH (disease) headings. (**b**) Alternative splicing of genes identified by the RNA sequencing analysis as having high ΔPSI values was tested by RT-PCR analysis using cardiac mRNA from rats of the indicated *RBM20* genotypes. i1, isoforms expressed at higher levels in wild-type rats than in mutant rats; i2, isoforms expressed at higher levels in mutant rats than in wild-type rats. Rbm20 exon 1 (5′) and exons 6 and 7 (3′) were amplified as controls; only the 5′ exon was present in the homozygous mutant. PCR fragments were sequence verified, as indicated in **Supplementary Figure 9**. Trdn, triadin; Sorbs1, sorbin and SH3 domain containing 1; Sh3kbp1, SH3-domain kinase binding protein 1. (**c**) Relative exon expression as determined by qRT-PCR using cardiac mRNA from rats of the indicated RBM20 genotypes. The regulation of LDB3 is opposite of all other genes tested with reduced inclusion of the alternative exon in homozygotes (–/–). The primers used are listed in **Supplementary Figure 8**. $^{*}P \le 0.05$, $^{***}P \le 0.001$, one-way ANOVA. $n = 3$ rats per group. All data are means ± s.e.m.

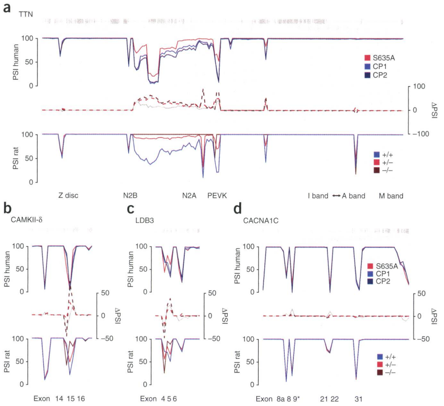

**Figure 6** Alignment of orthologous rat and human exons to compare RBM20-dependent isoform expression. (**a**–**d**) PSI scores for the RBM20-deficient individual (S635A) are indicated in red and those for two control subjects with DCM of unrelated cause (CP1 and CP2) are indicated in blue. The average PSI of three rats per genotype is provided below and is aligned to the human exon structure (wild type, blue; *RBM20* heterozygote, light red; *RBM20* homozygote, dark red). The resulting $\Delta$PSIs are shown for humans (gray; continuous line; S635A compared to the average of CP1 and CP2) and rat (dashed lines; light red indicates wild type compared to heterozygous rats, and dark red indicates wild type compared to homozygous rats). The $\Delta$PSI values show highly conserved deflections up (additional inclusion of exons) and down (exon skipping) in differentially spliced regions across species. Heterozygous rats show an intermediate level of alternative exon usage as compared to homozygotes and wild types. (**a**) TTN is predominantly spliced in the elastic region extending from the N2B to the PEVK region. The 5′ and 3′ regions are unaffected, as indicated by $\Delta$PSI curves approaching 0. Both alternatively spliced and constitutive regions are conserved between species. (**b**) CAMKII-δ exons 14–16 are mutually exclusive and are differentially regulated by RBM20, with exon 14 upregulated in the control individuals and exons 15 and 16 upregulated in the individuals with the S635A mutation (indicated by the crossing of PSI lines between exons 14 and 15). (**c**) LDB3 undergoes an analogous exon switch that affects exon 4 (included in the control individuals and wild-type rats and exons 5 and 6 (included in the S635A human and *Rbm20* mutant rats). (**d**) CACNA1C shows a conserved splicing pattern in human and rat for the majority of its alternatively spliced exons (8, 9* (an alternative exon 9 that can replace the standard exon 9), 22 and 31), but the RBM20-dependent differences in inclusion rates were small, with $\Delta$PSI < 10. $n = 3$ rats per genotype.

cardiomyopathy after cardiac transplantation who did not carry any mutations causing nonsynonymous amino acid changes in RBM20 (as determined by Sanger sequencing of the entire coding region). The individual with the heterozygous *RBM20* mutation had a shift in titin isoform expression, with a higher–molecular-weight titin isoform compared to the isoform present in the controls (**Fig. 4e**). This result is consistent with the rat studies, in which the larger titin isoform is expressed in heterozygous mutant rats (**Fig. 4e**). The two control subjects expressed the N2B and N2BA titin isoforms of lower molecular weight, similar to the pattern found in healthy individuals (**Fig. 4e**).

## RBM20 regulates select genes related to cardiac disease

We used deep sequencing of the rat and human cardiac transcriptomes to refine our analysis of RBM20-dependent splicing. High-throughput RNA sequencing generates information at both the gene and the exon level, facilitating the identification of additional candidate substrates, as well as the exon composition of RBM20-dependent isoforms. We analyzed the cardiac transcriptomes of wild-type rats and rats either homozygous or heterozygous for the *Rbm20*-null allele ($n = 3$ rats per group) and compared them to the transcriptomes of humans with DCM with and without mutations in *RBM20*. We used cardiac tissue from relatively young rats (2 months of age), before the onset of cardiomyopathy, to minimize any potential secondary effects of cardiomyopathy on alternative splicing that are not dependent on RBM20. We generated ~150 million sequencing reads per sample (100-bp paired-end reads; **Supplementary Fig. 7**) and found 31 genes with shared *RBM20*-genotype–dependent splicing between rats and humans (false discovery rate <5%), which is a significantly greater overlap than would be expected by chance ($P < 10^{-5}$). This RBM20-dependent gene network includes *TTN*, whose alternative splicing was expected based on our initial experiments and, thus, validated our approach. We further confirmed the RNA sequencing results by RT-PCR and qRT-PCR for *TTN* and for six additional genes (**Fig. 5** and **Supplementary Fig. 8**). A Gene Ontology analysis of the RBM20-driven network showed enrichment for genes encoding proteins involved in sarcomere organization and ion transport in the sarcoplasmic reticulum (**Fig. 5**, **Supplementary Tables 2–5** and **Supplementary Fig. 9**). The 31 genes showed additional enrichment for the NCBI Medical Subject Headings (MeSH) 'heart failure' and 'cardiomyopathy' (**Fig. 5** and **Supplementary Table 6**).

Several genes with RBM20-dependent isoform expression showed identical splicing patterns on the exon level across species (**Fig. 6**). *Ttn*, *Camk2d*, *Ldb3* and *Cacna1c* showed conserved differential splicing of orthologous exons between rat and human for the majority of their alternatively spliced exons (**Figs. 5** and **6**). Of these four genes, *Ttn* was the most extensively mis-spliced in rats and humans: long stretches of exons were affected, the majority of which were overrepresented in RBM20 deficiency. This result is consistent with the expression of larger titin protein isoforms in both Rbm20-deficient rats and the human subject with the RBM20 S635A variant (**Figs. 4** and **6**). The alternatively spliced exons reside in the elastic PEVK- and the immunoglobulin-rich region within the I band, which may help explain the increased distensibility of Rbm20-deficient sarcomeres[16].

To show direct binding of RBM20 to its target splice sites, we focused on the 5′ splice site at the start of titin's alternatively spliced I-band region. Using a retention assay, we were able to complement our crosslinking- and immunoprecipitation-derived RNA-binding data regarding the interaction of RBM20 and titin RNA: titin mRNA corresponding to a 5′ exon-intron junction that precedes an alternatively spliced I-band region interacts with a glutathione-S-transferase (GST)-RRM fusion protein containing the RNA recognition motif of RBM20, but not with the GST protein negative control (**Supplementary Fig. 10**).

For calcium/calmodulin-dependent protein kinase II δ (CaMKII-δ), *RBM20* mutation resulted in splicing out of exon 14 and the inclusion of exons 15 and 16 (**Fig. 6b**). This caused an isoform shift from CaMKII-δB to CaMKII-δA. It should be noted that exon 14 of CaMKII-δ contains its nuclear localization signal. A similar shift has been described in serine/arginine-rich splicing factor 1 (ASF, also called SF2)–deficient mice with altered excitation contraction coupling that leads to cardiac hypercontractility[23]. For the protein LDB3 (LIM domain binding 3), inclusion of exon 4 or exons 5 and 6 was mutually exclusive, and this differential inclusion was regulated by RBM20. Expression of exon 4 was downregulated in left ventricular tissue from Rbm20-deficient rats and in the individual with the *RBM20* missense mutation (**Fig. 6b**). For the calcium channel subunit Cacna1c (the α1C subunit of the L-type calcium channel), differential isoform expression has previously been related to arrhythmia[24]. We found that RBM20 mutation resulted in conserved changes in exon inclusion in this protein, although with smaller effects compared to the other three genes. This was assessed by the change in percentage spliced in (PSI) levels (**Fig. 6d**). Finally, rat RNA-Seq data validated the deletion of RBM20 exons, as only exon 1 was expressed at similar levels in wild-type and Rbm20-deficient (**Supplementary Fig. 11**). Furthermore, the intermediate levels in heterozygotes suggest the faithful representation of changes at the exon level in the RNA-Seq data set.

## DISCUSSION

RBM20 has recently been linked to human cardiac disease, with *RBM20* mutations present in 1.9–3% of individuals with idiopathic DCM[17,18], but the underlying mechanisms by which *RBM20* mutation causes pathology have been unclear. Our analysis of RBM20 function *in vivo* and at the molecular level helps resolve this issue and shows that it regulates cardiac isoform expression. Rbm20-deficient rats and humans with *RBM20* missense mutations both have cardiomyopathy with fibrosis and arrhythmia with sudden death. Our genome-wide transcriptomic analysis revealed a set of 31 RBM20-dependent genes, of which four (*TTN*, *CAMK2D*, *LDB3* and *CACNA1C*) showed conserved regulation between rats and humans down to the exon level. As implied by the characterization of its substrate profile, which includes alternatively spliced genes encoding structural, sarcomeric, enzymatic and signaling proteins, RBM20 regulates the isoform expression of genes related to diastolic function, sarcomere assembly and ion transport.

We verified the transcriptomics data using several methods, including the confirmation of concordant protein isoform and RNA isoform expression of rat and human titin. Because of a lack of sufficient human biopsy material, we focused our validation of the RNA-Seq data on the rat. RT-PCR and qRT-PCR analyses confirmed the differential isoform expression of 7 of the 14 genes that were identified as differentially spliced by RNA-Seq with a ΔPSI >10% dependent on RBM20. Our deep sequencing analysis with long (100 bp) paired-end reads that span exon borders enabled the detection of even small changes in isoform regulation with a statistical significance that cannot be obtained using RT-PCR (ΔPSI <10%; data not shown). Because *RBM20*-genotype–dependent isoform expression was consistent between species, we also considered genes with low ΔPSI values as part of the set of RBM20-regulated candidate genes.

Our analysis of titin-splice–deficient rats revealed a genomic deletion leading to a loss of function of the SR-related protein Rbm20. SR and SR-related proteins are highly conserved in metazoans[25], and their modular structure, with one or two RNA recognition motifs and an RS domain, is essential for RNA and protein interactions and recruitment of the splicing machinery[26]. About half of the more than 100 SR-related proteins identified to date have been implicated in splicing[2]. Regulation of alternative splicing can be achieved by temporal and spatial control of the tissue-specific expression of RS proteins or by post-translational modification of these proteins. Similar to its largest substrate, titin, Rbm20 is predominantly expressed in striated muscle (in contrast to most SR-related proteins, which are ubiquitously expressed), and we found that its expression in differentiating myocytes peaks as their sarcomeric structure is being built. The difference in the calculated size of Rbm20 of ~130 kDa and its migration during gel electrophoresis at ~180 kDa implies that it is post-translationally modified. Indeed, SR-related proteins are frequently regulated by phosphorylation, resulting in a sizeable difference between the calculated and observed molecular weights for many of these proteins[27]. The phosphorylation of SR-related proteins has previously been associated with re-import into the nucleus[27] and the regulation of alternative splicing[28]. We found that Rbm20 is localized to the nucleus throughout myogenic differentiation and did not detect it in the cytoplasm. This would suggest a function for RBM20 in the major (nuclear) spliceosome.

The availability of rat and human cardiac tissue for analysis of RBM20-dependent isoform expression provided several leads toward understanding the mechanism underlying RBM20-related pathology. This analysis suggests that RBM20 has a crucial role in multiple aspects of cardiac function through effects on genes involved in biomechanics (*TTN* and *TPM1*), ion homeostasis and electrical activity (*CAMK2D* and *CACNA1C*) and signal transduction (*CAMK2D* and *SPEN*). Furthermore, in addition to differential splicing of titin[6] and CAMKII-δ[29], changes in the isoform expression of LDB3 (also known as cypher) and CACNA1C have been shown to be related to dilated cardiomyopathy and arrhythmia, respectively[30,31].

Titin is a prominent target of RBM20, and *TTN* mutations are known to contribute to cardiomyopathy[6,32]. Although altered titin splicing is frequently found in cardiovascular disease[7,33–35], previously reported changes in titin isoform expression are rather minor, unlike the effects of the RBM20 mutation, which leads to the production of extremely large N2BA isoforms. These isoforms are predicted to cause an increase in titin-based elasticity that has implications for diastolic function and the Frank-Starling mechanism[16]. Furthermore, the reduced recoil associated with a flaccid titin filament could lead to a compensatory increase in collagen biosynthesis and ultimately to fibrosis.

Calcium handling is a crucial component of cardiac function and is affected in all knockouts of cardiac splice factors described to date. Deficiency in either serine/arginine-rich splicing factor 2 (SC35) or serine/arginine-rich splicing factor 10 (SRp38) leads to altered $Ca^{2+}$ release from the sarcoplasmic reticulum with downregulation of the ryanodine receptor 2 (RyR2)[36] and defective splicing of *TRDN* (encoding triadin)[37], respectively. As we also identified *TRDN* as a substrate of RBM20, both SRp38 and RBM20 seem to regulate splicing of this gene. Mutations in both *SRSF1* (encoding ASF, also known as SF2) and *RBM20* affect CAMKII-δ isoform expression. Mutations in either gene lead to the production of isoforms lacking a nuclear localization signal[23]. Such isoforms are mistargeted to the sarcolemma, causing not only excitation-contraction coupling defects[23] but also an increase in a propensity for ventricular tachycardia[38], similar to the phenotype we observed in Rbm20-deficient rats.

Mutations in LDB3 have previously been implicated in dilated cardiomyopathy[39,40]. Here we found that RBM20 mediates mutually exclusive inclusion of exon 4 or exons 5 and 6 of LDB3. Exon 4 of LDB3 has been implicated in binding to the glycolytic enzyme phosphoglucomutase 1 (PGM1), and mutations in exon 4 of *LDB3* have been shown to associate with DCM through impaired binding of PGM1 (ref. 41). We here show exon 4 of LDB3 to be downregulated in cardiac tissue from Rbm20-deficient rats and from the human subject with a *RBM20* missense mutation. This shift in LDB3 isoform expression would be expected to decrease the number of PGM1 binding sites, which could conceivably alter recruitment of the glycolytic enzyme PGM1 to the Z disk.

DCM is a leading cause of heart failure and cardiac transplantation in Western countries and has been extensively studied[42]. Although there is evidence of a substantial inherited contribution to DCM, and numerous causal genes for DCM have been identified, within affected families it is often the case that there is incomplete penetrance and a variable phenotype[43], and the causal gene is known in only a small subset of such families. Among the genes identified are those encoding sarcomeric contractile proteins, cytoskeletal proteins, nuclear membrane proteins, and the dystrophin-associated glycoprotein complex[44]. Notably, although >15% of human genetic diseases have been attributed to mutations in *cis*-acting splice elements. *RBM20* mutation is one of only a few examples of a *trans*-acting effect and establishes the regulation of alternative splicing as a hitherto unappreciated cause of cardiomyopathy[5,45].

In conclusion, our data suggest a model in which reduced activity of RBM20 results in altered isoform expression of proteins that maintain sarcomeric structure and cardiac function, among them titin, CAMKII-δ, LDB3 and CACNA1C. These changes may result in altered biomechanics, electrical activity and signal transduction that ultimately lead to cardiomyopathy, fibrosis, arrhythmia and sudden death. Our data highlight the key role of post-transcriptional regulation in physiological cardiac function and heart failure.

## METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

**Accession codes.** All rat and human RNA sequencing data have been submitted to the Sequencing Read Archive of the European Nucleotide Archive (ERP001301, http://www.ebi.ac.uk/ena/data/view/ERP001301). The GenBank accession numbers for human and rat RBM20 are EU822950 and EU562301, respectively.

*Note: Supplementary information is available on the Nature Medicine website.*

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AUTHOR CONTRIBUTIONS

M.L.G., N.H. and M.G. designed the research. W.G., S.S., M.H.R., M.L., T.G., H.M., H.S., S.L., A.M.P., V.D., P.V., S.K., B.G., L.T., V.R.-Z., T.A.H., K.W.S., G.W.D., P.T.E., C.A.M., B.S., R.F., A.P., C.O. and K.S. performed the research. W.G., S.S., M.L.G., H.S., M.H.R., M.L., T.G., H.M., S.L., A.M.P., V.D., P.V., S.K., B.G., L.T., V.R.-Z., T.A.H., K.W.S., G.W.D., P.T.E., C.A.M., B.S., R.F., A.P., C.O., K.S. and M.G. performed data analysis. W.G., T.G., H.M., H.S., S.L., A.M.P., V.D., P.V., S.K., B.G., L.T., V.R.-Z., T.A.H., K.W.S., G.W.D., P.T.E., C.A.M., B.S., R.F., A.P., C.O. and K.S. provided discussion and advice. V.R.-Z., A.P. and C.O. provided patient material. S.S., M.H.R., T.G., H.S. and M.G. performed the bioinformatics analysis. S.S., M.L.G., M.H.R., N.H. and M.G. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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# Adora2b-elicited *Per2* stabilization promotes a HIF-dependent metabolic switch crucial for myocardial adaptation to ischemia

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**Adenosine signaling has been implicated in cardiac adaptation to limited oxygen availability. In a wide search for adenosine receptor A2b (Adora2b)-elicited cardioadaptive responses, we identified the circadian rhythm protein period 2 (Per2) as an Adora2b target. Adora2b signaling led to Per2 stabilization during myocardial ischemia, and in this setting, *Per2*$^{-/-}$ mice had larger infarct sizes compared to wild-type mice and loss of the cardioprotection conferred by ischemic preconditioning. Metabolic studies uncovered a limited ability of ischemic hearts in *Per2*$^{-/-}$ mice to use carbohydrates for oxygen-efficient glycolysis. This impairment was caused by a failure to stabilize hypoxia-inducible factor-1α (Hif-1α). Moreover, stabilization of Per2 in the heart by exposing mice to intense light resulted in the transcriptional induction of glycolytic enzymes and Per2-dependent cardioprotection from ischemia. Together, these studies identify adenosine-elicited stabilization of Per2 in the control of HIF-dependent cardiac metabolism and ischemia tolerance and implicate Per2 stabilization as a potential new strategy for treating myocardial ischemia.**

Metabolic adaptation during environmental stress is currently an area of intense investigation because of the potential implications of metabolic alterations in human disease[1,2]. For example, myocardial ischemia leads to the activation of pathways directed toward enhancing myocardial oxygen efficiency[3]. A metabolic switch from a more 'energy-efficient' utilization of fatty acids to a more 'oxygen-efficient' utilization of glucose as the main source for energy generation is pivotal for allowing the myocardium to function under ischemic conditions[4,5].

Signaling by extracellular adenosine has been implicated in cellular adaptation to hypoxia[2]. In the extracellular compartment, adenosine is produced from the phosphohydrolysis of AMP by ecto-5′-nucleotidase (NT5E)[6] and signals through four adenosine receptors (adenosine receptor A1 (ADORA1), ADORA2A, ADORA2B and ADORA3)[7]. During conditions of hypoxia, adenosine generation is markedly enhanced, and the activation of adenosine receptors has a key role in counterbalancing the deleterious effects of hypoxia[1,8]. Particularly during conditions of myocardial ischemia, adenosine signaling events have been implicated in cardioprotection from ischemia. Similarly, cardioprotective responses elicited by ischemic preconditioning are abolished after pharmacological inhibition or genetic ablation of extracellular adenosine production or signaling[9]. Here we identify the circadian rhythm protein Per2 as a key mediator of Adora2b-elicited cardioprotection by enhancing the glycolytic capacity of the ischemic heart.

## RESULTS

### The circadian rhythm gene *Per2* as an Adora2b target gene

Previous studies have implicated adenosine receptor signaling in myocardial adaptation to ischemia or hypoxia in mice[10]. Here we studied these pathways in cardiac tissues obtained from patients with ischemic heart disease (average ejection fraction (EF%) of the left ventricle, 18.75; **Supplementary Tables 1** and **2**). Compared to cardiac tissues derived from healthy hearts, we found a selective induction of ADORA2B expression in diseased hearts (**Fig. 1a**). Together with previous studies in gene-targeted mice[10], these findings in human patients implicate extracellular adenosine signaling through ADORA2B in cardioprotection from ischemia.

Given the prominent role of adenosine receptor signaling in ischemic preconditioning, we performed microarray studies to compare the transcriptional responses elicited by ischemic preconditioning (**Supplementary Fig. 1**) in wild-type and *Adora2b*$^{-/-}$ mice (**Fig. 1b**, **Supplementary Figs. 2–4** and **Supplementary Table 3**). The gene with highest differential readout was *Per2*, encoding a circadian rhythm protein, which was exclusively upregulated in wild-type hearts. *Per2*, a member of the Period family of genes, is expressed in a circadian rhythm pattern in the suprachiasmatic nucleus[11]. In addition to *Per2*, the microarray studies also showed a similar regulatory pattern for *Per1*, whereas other circadian rhythm genes were not affected (**Supplementary Figs. 3–5** and **Supplementary Table 3**).

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We found that ischemic conditioning did not induce mRNA expression of the circadian control proteins Clock, Cryptochrome 1 (Cry1) or Timeless, and protein stabilization occurred in an Adora2b-independent manner (**Supplementary Fig. 5**). As studies in *Per1*$^{-/-}$ mice did not identify a functional role for Per1 in myocardial ischemia (**Supplementary Figs. 6** and **7**), we instead focused on Per2. We found that cardiac *Per2* mRNA was expressed in a circadian pattern, as measured over a 24-h zeitgeber period (measured according to zeitgeber time, ZT, where ZT0 denotes the time of lights on and ZT12 indicates the time of lights off), and that this pattern was abolished in *Adora2b*$^{-/-}$ mice. Moreover, the induction of Per2 mRNA and protein expression after ischemic preconditioning was abolished in the hearts from *Adora2b*$^{-/-}$ mice (**Fig. 1c–f**, **Supplementary Fig. 1** and **Supplementary Table 4**). Studies in isolated cardiac myocytes exposed to *in vitro* hypoxic preconditioning (HPC; **Supplementary Fig. 8**) showed enhanced *Adora2b* and *Per2* mRNA expression in wild-type but not *Adora2b*$^{-/-}$ myocytes (**Fig. 1g**). Moreover, we found elevated PER2 mRNA and protein expression in the cardiac

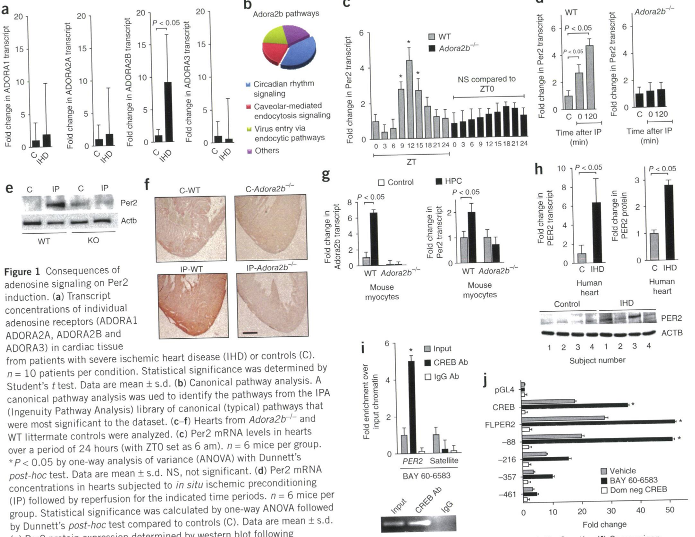


**Figure 1** Consequences of adenosine signaling on Per2 induction. (**a**) Transcript concentrations of individual adenosine receptors (ADORA1 ADORA2A, ADORA2B and ADORA3) in cardiac tissue from patients with severe ischemic heart disease (IHD) or controls (C). $n = 10$ patients per condition. Statistical significance was determined by Student's *t* test. Data are mean ± s.d. (**b**) Canonical pathway analysis. A canonical pathway analysis was ued to identify the pathways from the IPA (Ingenuity Pathway Analysis) library of canonical (typical) pathways that were most significant to the dataset. (**c**–**f**) Hearts from *Adora2b*$^{-/-}$ and WT littermate controls were analyzed. (**c**) Per2 mRNA levels in hearts over a period of 24 hours (with ZT0 set as 6 am). $n = 6$ mice per group. $*P < 0.05$ by one-way analysis of variance (ANOVA) with Dunnett's *post-hoc* test. Data are mean ± s.d. NS, not significant. (**d**) Per2 mRNA concentrations in hearts subjected to *in situ* ischemic preconditioning (IP) followed by reperfusion for the indicated time periods. $n = 6$ mice per group. Statistical significance was calculated by one-way ANOVA followed by Dunnett's *post-hoc* test compared to controls (C). Data are mean ± s.d. (**e**) Per2 protein expression determined by western blot following ischemic preconditioning without reperfusion. WT, wild type; KO, *Adora2b*$^{-/-}$. One representative blot of three is shown. Actb, β-actin. (**f**) Comparison of the immunoreactivity for Per2 in preconditioned cardiac tissue (IP-WT and IP-*Adora2b*$^{-/-}$) or sham-operated control tissue (C-WT and C-*Adora2b*$^{-/-}$). One representative image of three for each condition is shown. Scale bar, 100 μm. (**g**) Adora2b and Per2 mRNA concentrations in isolated adult mouse cardiomyocytes from wild-type or *Adora2b*$^{-/-}$ mice after *in vitro* exposure to HPC. $n = 4$ hearts per group. Statistical significance was calculated by Student's *t* test. Data are mean ± s.d. (**h**) PER2 mRNA (left) or protein (right) concentrations in cardiac tissues from patients with severe ischemic heart disease (IHD) or controls (C). A representative immunoblot for PER2 protein from the indicated subjects is shown below (see **Supplementary Fig. 9**). ACTB (β-actin) was used a loading control. $n = 10$ patients per condition. Statistical significance was calculated by Student's *t* test. Data are mean ± s.d. (**i**) Chromatin immunopreciptitation analysis to detect CREB protein binding to the *PER2* promoter using HMEC-1 cells treated with BAY 60-6583 for 20 min. Real-time RT-PCR for human *PER2* promoter or satellite DNA (negative control) (top). Products obtained by PCR for the *PER2* promoter analyzed on a 1% agarose gel (bottom). Data are mean ± s.d. $n = 3$. $*P < 0.05$ by Student's *t* test. Ab, antibody; IgG, immunoglobulin G. (**j**) Luciferase activity measured in transfected HMEC-1 cells with a full-length *PER2* promoter construct (FLPER2) or the indicated truncated versions, subcloned into the pGL4 luciferase reporter vector. The pGL4 vector used alone served as a negative control. As a positive control, a pMetLuc reporter vector containing a specific promoter for CRE was used (CREB). The cells were treated with vehicle or BAY 60-6583 or were cotransfected with a dominant negative CREB (Dom neg CREB) construct, as indicated. Schematic diagrams of the truncated versions of the *PER2* promoter, as well as the location of putative CREB binding sites, are shown in **Supplementary Figure 11**. $*P < 0.05$ by one-way ANOVA with Dunnett's *post-hoc* test for luciferase activity over baseline. Data are mean ± s.d. $n = 6$.

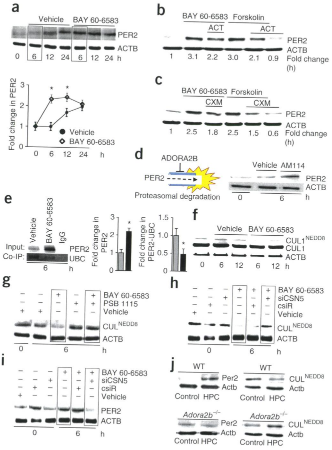


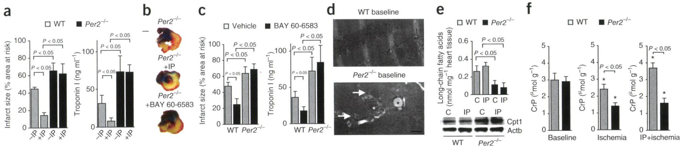
**Figure 2** Influence of transcriptional, translational and post-translational mechanisms on Per2 protein expression. (**a**) Immunoblot analysis of PER2 protein using synchronized HMEC-1 cells treated with vehicle or BAY 60-6583 for the indicated time periods. One of three representative experiments is shown and is quantified below; boxed protein signals indicate the largest difference in PER2 signal strength between controls and cells treated with BAY 60-6583. $*P < 0.05$ by Student's *t*-test. Data are mean ± s.d. $n = 3$. (**b**,**c**) Immunoblot analysis of PER2 protein in synchronized HMEC-1 cells treated with vehicle, BAY 60-6583, or forskolin with or without actinomycin (ACT) (**b**) or cycloheximide (CXM) (**c**). Protein signals were quantified using densitometry, and the fold change over control is given below the blots. The effectiveness of ACT and CXM is shown in **Supplementary Figure 12a**,**b**. (**d**) Proposed model of adenosine-dependent alteration on post-translational PER2 protein stability (left). Immunoblot analysis of PER2 protein in synchronized HMEC-1 cells with or without addition of the proteasome inhibitor AM114 (right). One of three representative blots is shown. (**e**) Protein lysates were obtained from synchronized HMEC-1 cells treated with vehicle or BAY 60-6583 and were immunoprecipitated with PER2 antibody. The presence of ubiquitin (UBC) in the immunoprecipitate was determined by immunoblot analysis (left). Co-IP, coimmunoprecipitation. One representative blot of three is shown. Changes in protein concentration of PER2 (middle) and coimmunoprecipitated UBC (right) after BAY 60-6583 treatment, as determined by densitometry. $*P < 0.05$ by Student's *t* test. $n = 3$. Data are mean ± s.d. (**f**) Immunoblot analysis for total CUL1 or neddylated CUL1 (CUL1$^{\text{NEDD8}}$) in synchronized HMEC-1 cells treated with vehicle or BAY 60-6583. One of three representative blots is shown. (**g**) Immunoblot analysis for CUL$^{\text{NEDD8}}$ in synchronized HMEC-1 cells treated with BAY 60-6583 alone or after pretreatment with the ADORA2B antagonist PSB 1115. One of three representative experiments is shown. (**h**,**i**) Immunoblot analysis for CUL$^{\text{NEDD8}}$ (**h**) and PER2 (**i**) in synchronized HMEC-1 cells after siRNA knockdown of CSN5 (siCSN5) or treatment with nonspecific control siRNA (csiR). The cells were treated with vehicle or BAY 60-6583 as indicated. One of three representative experiments is shown. (**j**) Immunoblot analysis for CUL$^{\text{NEDD8}}$ and Per2 in cardiac myocytes isolated from wild-type or *Adora2b*$^{-/-}$ mice exposed to HPC or control conditions. One representative blot of three independent experiments is shown. $n = 1$ mouse per experiment.

tissues of patients with ischemic heart disease ($n = 10$ per group; **Fig. 1h**, **Supplementary Fig. 9** and **Supplementary Table 1**). Studies in a cultured human endothelial cell line (HMEC-1; **Supplementary Fig. 10**) revealed binding of cAMP response element binding protein (CREB) to the PER2 promoter after treatment with an ADORA2B agonist, BAY 60-6583 (ref. 10) (**Fig. 1i**). Studies using truncated PER2 promoter constructs identified a CREB binding site that is responsible for the ADORA2B inducibility of the *PER2* promoter and that is conserved between mice and humans (**Fig. 1j** and **Supplementary Fig. 11**). Together, these studies indicate that ADORA2B signaling transcriptionally induces PER2 mRNA and protein expression.

## ADORA2B attenuates proteasomal degradation of PER2

The rapid kinetics of PER2 accumulation after adenosine receptor activation together with previous reports demonstrating post-translational regulation of PER2 expression[12] prompted us to investigate post-translational mechanisms of ADORA2B-dependent regulation of PER2. As the difference in PER2 expression between non-treated control and ADORA2B-agonist–treated HMEC-1 cells was at a maximum at 6 h after treatment (**Fig. 2a**), we examined the influence of the inhibition of transcription by actinomycin or the inhibition of translation with cycloheximide at this time point (**Fig. 2b**,**c** and **Supplementary Fig. 12a**). These experiments provided evidence for a combination of transcriptional and post-translational mechanisms in ADORA2B-dependent regulation of PER2 expression: whereas actinomycin or cycloheximide effectively inhibited transcription or translation (**Supplementary Fig. 12a**), respectively, ADORA2B-agonist treatment was still able to enhance PER2 protein stabilization with either treatment. However, the highest level of PER2 protein accumulation was seen with ADORA2B-agonist treatment alone.

We next investigated whether ADORA2B signaling could interfere with proteasomal degradation of PER2 (**Fig. 2d**). We pretreated HMEC-1 cells with the proteasome inhibitor AM114, which resulted in prominent PER2 stabilization (**Fig. 2d**). Previous studies have indicated that post-translational degradation of Per2 involves the SCF E3 ubiquitin ligase complex, resulting in polyubiquitination of Per2 and subsequent degradation by the 26S proteasome[13]. The SCF complex is active only when cullin 1 (CUL1) is covalently modified by the ubiquitin-like protein NEDD8 (ref. 14). Indeed, immunoprecipitation of PER2 and immunoblotting for ubiquitin showed attenuated PER2 ubiquitination after treatment with the ADORA2B agonist BAY 60-6583 (**Fig. 2e**). Given that ADORA2B signaling has been shown to deneddylate CUL1 (ref. 15), we investigated ADORA2B-dependent alterations of the neddylation status of CUL1. We confirmed that deneddylation of CUL1 was enhanced after treatment with the ADORA2B agonist (**Fig. 2f** and **Supplementary Fig. 12c**,**d**). Furthermore, pretreatment with an ADORA2B antagonist (PSB1115) blocked ADORA2B-agonist–dependent cullin deneddylation (**Fig. 2g**). Cullin deneddylation is accomplished through interaction with subunits of the COP9 signalosome (for example, CSN5, also known as JAB1)[16]. We therefore examined whether decreasing CSN5 expression (and, thereby, inhibition of cullin deneddylation) might influence



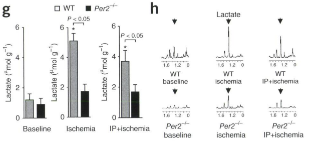


**Figure 3** Functional role of Per2 during myocardial ischemia and ischemic preconditioning. (**a**) Infarct sizes and troponin I concentrations in plasma samples from *Per2*$^{-/-}$ mice and WT littermate controls that were exposed to *in situ* myocardial ischemia followed by reperfusion or received ischemic preconditioning or treatment with BAY 60-6583 before myocardial ischemia. Infarct sizes are expressed as the percent of the area at risk that was exposed to myocardial ischemia. Data are mean ± s.d. $n = 6$ mice per group. Statistical significance was calculated by two-factor ANOVA with Bonferroni's *post-hoc* test. −IP, ischemia and reperfusion only; +IP, ischemic preconditioning. (**b**) Representative infarct staining in hearts from *Per2*$^{-/-}$ mice exposed to ischemia and reperfusion only (top) or to ischemic preconditioning (middle) or BAY 60-6583 (bottom) before myocardial ischemia (blue, retrograde Evan's blue staining; red and white, area at risk; white, infarcted tissue). Scale bar, 50 μm. (**c**) Infarct sizes and troponin I concentrations in plasma samples from *Per2*$^{-/-}$ mice or WT littermate controls treated with either vehicle or BAY 60-6583. Data are mean ± s.d. $n = 6$ mice per group. Statistical significance was calculated by two-factor ANOVA with Bonferroni's *post-hoc* test. (**d**) Electron microscopy of wild-type and *Per2*$^{-/-}$ heart tissue. At baseline, wild-type and *Per2*$^{-/-}$ mice showed normal sarcolemmal structures, however, in some areas, the *Per2*$^{-/-}$ tissue showed enhanced glycogen content (white arrow), swollen mitochondria (white asterisk) and lipid accumulation within mitochondria (black asterisk). Scale bar, 500 nm. (**e**) Long-chain fatty acid concentrations (top) and immunoblot analysis of Cpt1 (bottom) in heart tissue from *Per2*$^{-/-}$ mice and WT littermate controls subjected to *in situ* ischemic preconditioning. Data are mean ± s.d. $n = 3$ mice per group. Statistical significance was calculated by Student's *t* test compared to control mice without ischemic preconditioning (C). One representative blot of three is shown. (**f–h**) *Per2*$^{-/-}$ mice and WT littermate controls were exposed to *in situ* myocardial ischemia with or without ischemic preconditioning. (**f**) Creatine phosphate (CrP) concentrations, as determined by NMR. (**g,h**) Lactate concentrations and the corresponding NMR spectra. $n = 3$ mice per group. $^*P < 0.05$ by Student's *t* test compared to baseline. Data are mean ± s.d.

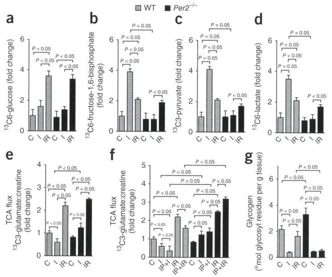
ADORA2B-dependent stabilization of PER2. Cullin deneddylation induced by BAY 60-6583 was attenuated in HMEC-1 cells that had been pretreated with CSN5-targeted siRNA (**Fig. 2h**, **Supplementary Fig. 12e,f** and **Supplementary Table 5**). Moreover, cells treated with CSN5-targeted siRNA lost their ability to stabilize PER2 in response to BAY 60-6583 (**Fig. 2i**). We then tested isolated adult cardiac myocytes from wild-type and *Adora2b*$^{-/-}$ mice. Exposure to HPC was associated with Per2 stabilization in wild-type but not *Adora2b*$^{-/-}$ myocytes. Similarly, HPC-induced cullin deneddylation was seen in wild-type but not *Adora2b*$^{-/-}$ myocytes (**Fig. 2j**). Taken together, these results indicate that ADORA2B signaling increases PER2 expression by effects on both *PER2* transcription and PER2 protein stability.

## Impaired myocardial adaptation to ischemia in *Per2*$^{-/-}$ mice

We next studied *Per2* gene-targeted mice that have a deletion of the entire PAS-B domain (**Supplementary Fig. 13a**)[11]. *Per2*$^{-/-}$ mice had increased tissue injury after myocardial ischemia and an abolishment of the cardioprotection conferred by ischemic preconditioning (**Fig. 3a,b**). Moreover, treatment with BAY 60-6583 led to a significant reduction of the size of infarcted tissue and the concentration of troponin I in plasma samples from wild-type but not *Per2*$^{-/-}$ mice, indicating that Adora2b-dependent cardioprotection is abolished in *Per2*$^{-/-}$ mice (**Fig. 3b,c**). Baseline electron microscopic imaging of the cardiac ultrastructure in *Per2*$^{-/-}$ mice showed isolated mitochondrial swelling and glycogen accumulation, with no major structural alterations of the myofibrillar apparatus (**Fig. 3d**). Baseline cardiac glycogen concentrations were elevated (**Supplementary Fig. 13b**), whereas long-chain fatty acid concentrations were lower, in *Per2*$^{-/-}$ compared to wild-type mice (**Supplementary Fig. 13c**). Consistent with these findings, *Per2*$^{-/-}$ mice had elevated protein concentrations of glycogen synthase 1 (Gys1) (**Supplementary Fig. 13d**) and carnitine palmitoyltransferase 1 (Cpt) (**Supplementary Fig. 13e**) compared to wild-type mice. However, baseline cardiac function assessed by echocardiography was unaltered in *Per2*$^{-/-}$ mice (**Supplementary Fig. 13f**). Consistent with recent studies of the role of Per2 in fatty acid metabolism[17], we found that either at baseline or after ischemic preconditioning, *Per2*$^{-/-}$ mice had decreased amounts of long-chain fatty acids and increased amounts of Cpt1 protein compared to wild-type mice (**Fig. 3e**). Because these studies indicate that *Per2*$^{-/-}$ hearts have enhanced fatty acid metabolism at baseline as well as after myocardial ischemia, we used nuclear magnetic resonance (NMR) studies to characterize the metabolic role of Per2. We exposed *Per2*$^{-/-}$ or wild-type mice to ischemia alone (60 min) or to a protocol in which ischemia was preceded by ischemic preconditioning. Although baseline creatine phosphate concentrations in cardiac tissue were similar in all groups, ischemia-associated creatine phosphate depletion was significantly enhanced in *Per2*$^{-/-}$ mice compared to wild-type mice, and the conservation of creatine phosphate concentrations by ischemic preconditioning was abolished in *Per2*$^{-/-}$ mice (**Fig. 3f**). Parallel measurements of lactate concentrations showed that ischemia-induced increases in the concentration of cardiac lactate were attenuated in *Per2*$^{-/-}$ mice (**Fig. 3g,h**), indicating a role for Per2 in the glycolytic utilization of carbohydrates during myocardial ischemia.

## Impaired cardiac glycolysis during ischemia in *Per2*$^{-/-}$ mice

Ischemic-preconditioning elicited an induction of glycolytic enzyme mRNA expression in the hearts of wild-type mice that was abolished in *Per2*$^{-/-}$ mice (**Supplementary Fig. 14** and **Supplementary Table 6**). We used liquid chromatography–tandem mass spectrometry studies



**h**

| | Gluc | Lac | F1,6bp | Pyr | TCA | Glycogen |
|---|---|---|---|---|---|---|
| WT+I | ↔ | ↑↑↑ | ↑↑↑ | ↑↑↑ | ↓ | ↓↓↓ |
| *Per2*$^{-/-}$ +I | ↔ | ↔ | ↔ | ↔ | ↑ | ↓↓↓ |
| WT+IP+I | ↔ | ↑↑ | ↑↑ | ↑↑ | ↓↓ | ↓↓ |
| *Per2*$^{-/-}$ +IP+I | ↔ | ↔ | ↔ | ↔ | ↑ | ↓↓↓ |
| WT+IR | ↑↑ | ↑ | ↑ | ↑ | ↑ | ↓ |
| *Per2*$^{-/-}$ +IR | ↑↑ | ↑ | ↑ | ↑ | ↑ | ↓↓↓ |
| WT+IP+IR | ↑↑↑ | ↔ | ↔ | ↔ | ↔ | ↔ |
| *Per2*$^{-/-}$ +IP+IR | ↑↑ | ↑ | ↑ | ↑ | ↑ | ↓↓↓ |

**Figure 4** Consequences of Per2 deficiency on cardiac metabolism during myocardial ischemia and reperfusion. (**a**–**g**) Metabolic parameters in *Per2*$^{-/-}$ mice and WT littermate controls exposed to *in situ* myocardial ischemia alone or to ischemia preceded by ischemic preconditioning. Both conditions were analyzed with and without reperfusion. For conditions without reperfusion, $^{13}$C-glucose was administered before ischemia, and with reperfusion, $^{13}$C-glucose was given at the onset of reperfusion. C, control; I, ischemia; IR, ischemia and reperfusion; IP, ischemic preconditioning. Data are mean ± s.d., $n = 3$ mice per group. *P* values were calculated by two-factor analysis of variance with Bonferroni's post-hoc test. Shown are $^{13}$C6-glucose concentrations (**a**), $^{13}$C6-fructose-1,6-bisphosphate concentrations (**b**), $^{13}$C3-pyruvate concentrations (**c**), $^{13}$C6-lactate concentrations (**d**),TCA cycle flux rates determined by the ratio of $^{13}$C3-glutamate to total creatine (**e**,**f**), and glycogen concentrations (**g**). Data are mean ± s.d., $n = 3$ mice per group. Statistical significance was calculated by two-factor ANOVA with Bonferroni's *post-hoc* test. (**h**) Summary of key metabolites of all experimental conditions in *Per2*$^{-/-}$ mice and WT littermate controls. Arrows indicate changes in experimental groups compared to their untreated control group. ↔, black, no changes; ↑ or ↓, black, slight increase or decrease compared to control; ↑↑ or ↓↓, black, strong increase or decrease compared to control; ↑↑↑ or ↓↓↓, black, very strong increase or decrease compared to control. Red arrows indicate differences between *Per2*$^{-/-}$ mice with the indicated treatments and equivalently-treated wild-type mice. WT, wildtype, I, ischemia alone; IP, ischemic preconditioning; IR, ischemia followed by reperfusion; Gluc, $^{13}$C6- glucose; Lac, $^{13}$C6-lactate; F1,6p, $^{13}$C6-fructose-1,6-bisphosphate, Pyr, $^{13}$C3-pyruvate; TCA, tricarboxylic acid cycle flux.

after the infusion of $^{13}$C-glucose into wild-type and *Per2*$^{-/-}$ mice to assess glucose metabolism during ischemia or reperfusion. Although we observed no differences between these mice in $^{13}$C-glucose uptake at baseline, during ischemia or at reperfusion (**Fig. 4a** and **Supplementary Fig. 15a**), a detailed analysis of the glycolytic flux revealed that ischemia elicited an increase of $^{13}$C-fructose-1,6-bisphosphate concentrations in wild-type but not *Per2*$^{-/-}$ mice, indicating that hypoxia-elicited enhancement of glycolysis during ischemia involves Per2 (**Fig. 4b** and **Supplementary Fig. 15b**). Similarly, ischemia induced elevations of $^{13}$C-pyruvate and $^{13}$C-lactate concentrations in wild-type but not *Per2*$^{-/-}$ mice (**Fig. 4c**,**d** and **Supplementary Fig. 15c**). In addition, whereas ischemia in wild-type mice attenuated the tricarboxylic acid (TCA) cycle flux of $^{13}$C-glucose, the TCA cycle flux of $^{13}$C-glucose in *Per2*$^{-/-}$ mice was increased during ischemia (**Fig. 4e**). Finally, ischemic preconditioning of wild-type mice was associated with an additional reduction in TCA cycle flux, a reduction that was abolished in *Per2*$^{-/-}$ mice (**Fig. 4f** and **Supplementary Fig. 15d**).

Although glycolytic utilization of carbohydrates is a key adaptive mechanism during ischemia[18], increased glycolysis during reperfusion is considered detrimental, as it frequently indicates mitochondrial dysfunction[19]. Indeed, tissue-reperfusion–attenuated glycolysis was associated with a reduced production of $^{13}$C-fructose-1,6-biosphosphate, $^{13}$C-pyruvate and $^{13}$C-lactate in wild-type mice (**Fig. 4b–d**). Although ischemia alone did not enhance glycolysis in *Per2*$^{-/-}$ mice, the glycolytic flux in these mice was increased during reperfusion (**Fig. 4e**,**f**). In wild-type mice, ischemic preconditioning led to a further reduction of glycolysis after reperfusion compared to ischemia that was not preceded by ischemic preconditioning, consistent with previous results[20], and to a restoration of glucose metabolism to a level comparable to that seen under baseline conditions (**Fig. 4f** and **Supplementary Fig. 15b–d**). In contrast, *Per2*$^{-/-}$ mice maintained lactate production after ischemia preceded by ischemic preconditioning, indicating an uncoupling of glycolysis from glucose oxidation, which is a sign of mitochondrial dysfunction[21] (**Supplementary Fig. 15c**). As we did not observe differences in glucose uptake between wild-type and *Per2*$^{-/-}$ mice during ischemia or reperfusion (**Fig. 4a**), we next analyzed the effect of metabolic changes on cardiac glycogen concentrations. Ischemia significantly reduced glycogen storage in both wild-type and *Per2*$^{-/-}$ mice, despite the fact that *Per2*$^{-/-}$ mice had a higher glycogen concentration at baseline (**Fig. 4g** and **Supplementary Fig. 15e**). Whereas reperfusion led to the restoration of glycogen concentrations in wild-type mice, this effect was abolished in *Per2*$^{-/-}$ mice. Together, these data indicate that *Per2*$^{-/-}$ mice are severely compromised in effectively using carbohydrates during ischemia or reperfusion (**Fig. 4h** and **Supplementary Table 7**).

## Hif-1α as a link to Per2 regulated cardiac metabolism

Ischemic preconditioning of wild-type mice was associated with a robust induction of glycolytic enzymes that was completely abolished in *Per2*$^{-/-}$ mice (**Supplementary Table 6**), prompting us to study the transcriptional mechanisms involved in this response (**Fig. 5**). Based on the notion that Hif-1α has a key role in the transcriptional control of the glycolytic pathway[22], we used a Hif-1α reporter mouse[23] to investigate the functional status of Hif-1α. Notably, over the course of a 24-h time period, we found circadian patterns for the concentrations of cardiac Hif-1α protein (**Fig. 5a**), Hif-1.1 and Hif-1.2 isoform mRNAs, and the glycolytic enzymes pyruvate dehydrogenase kinase, isozyme 1 (Pdk1) and lactate dehydrogenase (Ldh) in these mice (**Supplementary Fig. 16**). This was similar to the circadian pattern of cardiac Per2 protein observed in Per2 reporter mice (**Fig. 5a**) and of Per2 transcripts in hearts from wild-type mice (**Fig. 1c**). In Hif-1α reporter mice in which the *Per2* knockout allele had been introduced, Hif-1α cycling was abolished (**Fig. 5a**). In addition, the stabilization of Hif-1α by ischemic preconditioning seen in wild-type mice was lost in *Per2*$^{-/-}$ mice (**Fig. 5b**). Similarly, hypoxic Hif-1α stabilization was abolished in isolated myocytes from *Per2*$^{-/-}$ mice (**Fig. 5d**). Ischemic preconditioning of the Hif-1α reporter mice was associated

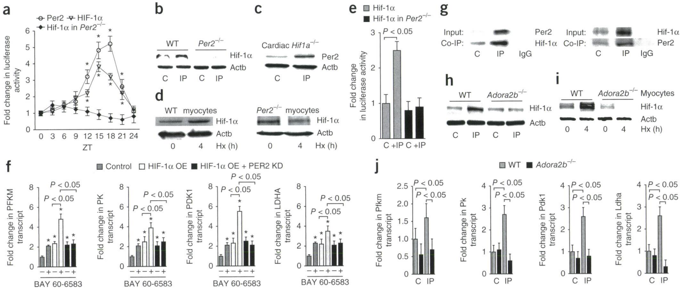


**Figure 5** Hif-1α functions as a link between adenosine-mediated Per2 signaling and metabolism. (**a**) Luciferase activity measured in the area at risk of hearts over a 24-h period (with ZT0 set as 6 am) from Hif-1α reporter mice, Per2 reporter mice, and *Per2*$^{-/-}$ mice containing the HIF-1α reporter (Hif-1α in *Per2*$^{-/-}$). *$P < 0.05$ by one-way ANOVA with Dunnett's *post-hoc* test compared to baseline. Data are mean ± s.d. $n = 3$ mice per group. (**b**,**c**) Immunoblot analysis for Hif-1α or Per2 in *Per2*$^{-/-}$ mice or mice with cardiac-specific knockout of *Hif1a* (cardiac *Hif1a*$^{-/-}$) subjected to ischemic preconditioning compared to untreated controls (C). Cardiac tissue in the area at risk was used. One representative blot of three is shown. (**d**) Immunoblot analysis for Hif-1α in isolated adult cardiomyocytes from wild-type or *Per2*$^{-/-}$ mice exposed to ambient hypoxia (Hx) for the indicated periods of time. One representative blot of three is shown. (**e**) Luciferase activity (as a readout of Hif-1α expression) in the area at risk of mice of the indicated genotypes exposed to myocardial ischemia-reperfusion with or without ischemic preconditioning. Data are mean ± s.d. $n = 4$ mice per group. Statistical significance was calculated by two-factor ANOVA with Bonferroni's *post-hoc* test. (**f**) Transcriptional regulation of glycolytic enzymes in control HMEC-1 cells or HMEC-1 cells overexpressing oxygen-stable HIF-1α (HIF-1α OE) without or with siRNA-mediated PER2 knockdown (HIF-1α OE + PER2 KD). Cells were also treated with vehicle or BAY 60-6583 as indicated. Shown are the transcript concentrations of phosphofructokinase-m (PFKM), pyruvate kinase (PK), pyruvate dehydrogenase kinase 1 (PDK1) and lactate dehydrogenase a (LDHA). Data are mean ± s.d., $n = 3$. Statistical significance was calculated by one-way ANOVA with Dunnett's *post-hoc* test. (**g**) Coimmunoprecipitation of Per2 and HIF-1α. Protein lysates from the hearts of wild-type mice subjected to ischemic preconditioning were immunoprecipitated with either Hif-1α or Per2 antibody, and the presence of the other protein in the immunoprecipitates was detected by immunoblotting. One representative blot of three is shown. (**h**,**i**) Immunoblotting for Hif-1α in hearts or isolated myocytes from wild-type or *Adora2b*$^{-/-}$ mice exposed to ischemic preconditioning or ambient hypoxia. One representative blot of three is shown. (**j**) Transcript concentrations of Pfkm, Pk, Pdk1 and Ldha in the hearts from wild-type and *Adora2b*$^{-/-}$ mice with or without ischemic preconditioning. Data are mean ± s.d. $n = 3$ mice per group. Statistical significance was calculated by one-way ANOVA with Dunnett's *post-hoc* test.

with increased reporter activity, which was abolished by genetic deletion of *Per2* (**Fig. 5e**). These data indicate that Hif-1α stabilization by hypoxia or ischemia depends on Per2 function. We next tested whether HIF-1α was reciprocally required for normal regulation of Per-2 expression. Mice in which the gene encoding Hif-1α was inducibly deleted in their cardiac myocytes (**Fig. 5c** and **Supplementary Fig. 17**) retained their ability to stabilize Per2 by ischemic preconditioning (**Fig. 5c**). Taken together, these data indicate that although Per2 is needed for the hypoxic regulation of Hif-1α, Per2 stabilization is not dependent on Hif-1α in cardiac tissue.

We next assessed the transcriptional regulation of glycolytic enzymes in oxygen-stable HIF-1α-overexpressing HMEC-1 cells[24] with or without siRNA-mediated PER2 knockdown. Oxygen-stable HIF-1α expression or treatment with the ADORA2B agonist BAY 60-6583 led to elevated glycolytic enzyme mRNA expression. Treatment with BAY 60-6583 further enhanced this elevation in oxygen-stable HIF-1α-overexpressing cells, an effect which was abolished by *PER2* knockdown (**Fig. 5f**). Additional studies using coimmunoprecipitation indicated a direct protein-protein interaction between Hif-1α and Per2 in cardiac tissues from wild-type mice after exposure to ischemic preconditioning (**Fig. 5g**).

As our results show that Adora2b signaling has a crucial role in the transcriptional induction and protein stability of Per2, we next examined Hif-1α concentrations in the hearts of *Adora2b*$^{-/-}$ mice. Similarly to our findings in *Per2*$^{-/-}$ mice, we observed lower expression of Hif-1.1 and Hif-1.2 isoform mRNA in *Adora2b*$^{-/-}$ mice compared to wild-type mice, as well as abolished circadian expression over a 24-h period (**Supplementary Figs. 16 and 18**). Moreover, ischemia or hypoxia-induced stabilization of Hif-1α was abolished in *Adora2b*$^{-/-}$ mice (**Fig. 5h,i**). We also observed a defect in the transcriptional induction of glycolytic enzymes by cardiac ischemic preconditioning in *Adora2b*$^{-/-}$ mice, similar to that seen in *Per2*$^{-/-}$ mice (**Fig. 5j**) Together, these data indicate that Adora2b-dependent control of Per2 has a key role in the hypoxia-elicited induction of the glycolytic machinery during myocardial ischemia.

## Light-induced cardiac Per2 mediates cardioprotection

We next attempted to achieve enhanced cardiac Per2 stabilization using light exposure[25,26]. We exposed mice to light with the same intensity as daylight (13,000 lx), which was achieved by using a light box with an ultraviolet filter, over a period of 4h and assessed their cardiac Per2 protein concentrations (**Fig. 6a**). We found time-dependent increases in cardiac Per2 concentrations compared to mice maintained at room light (200 lx) over the same time period. The same accumulation of cardiac Per2 as seen with 13,000 lx was normally achieved after a 12-h period at room light (**Fig. 6b** and **Supplementary Fig. 19a**). Exposure to illumination with an equivalent intensity to that of

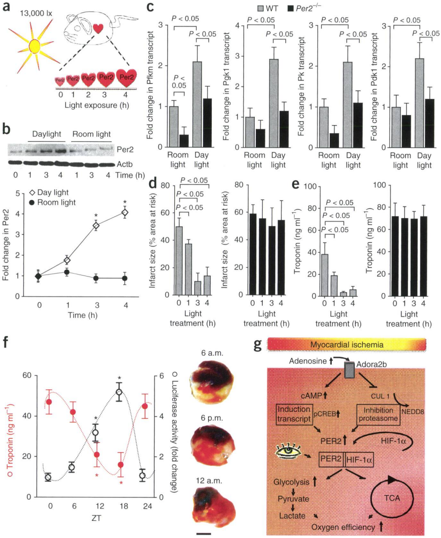


**Figure 6** Light-induced stabilization of cardiac Per2 expression provides potent protection from myocardial ischemia. (**a**) Experimental model for studying light-induced stabilization of cardiac Per2. (**b**) Immunoblotting for Per2 in the hearts of wild-type mice after exposure to 12 h of darkness and then to indicated time periods of intense light (13,000 lx; daylight) compared to control mice maintained at room light (top). Quantitation of the results by densitometry (below). $n = 4$ mice per group. $*P < 0.05$ by Student's $t$ test. Data are mean ± s.d. (**c**) Transcript concentrations of Pfkm, Pgk1, Pk and Pdk1 in the hearts of *Per2*$^{-/-}$or WT littermate control mice after 4 h of daylight exposure. $n = 4$ mice per condition. Statistical significance was calculated by one-way ANOVA with Dunnett's *post-hoc* test. Data are mean ± s.d. (**d**,**e**) Myocardial injury, as assessed by infarct size (**d**) or measurement of troponin I plasma concentrations (**e**), in *Per2*$^{-/-}$or WT littermate control mice exposed to light over the indicated time periods followed by exposure to *in situ* myocardial ischemia-reperfusion. $n = 6$ mice per group. Statistical significance was calculated by two-factor ANOVA with Bonferroni's *post-hoc* test. Data are mean ± s.d. (**f**) Troponin I in wild-type mice subjected to *in situ* myocardial ischemia reperfusion and Per2 expression as measured in hearts of untreated Per2 reporter mice at various times of day (with ZT0 set as 6 am) over a 24-h time period (left). Representative images of infarcts in mice subjected to myocardial ischemia-reperfusion at the indicated times of day (blue, retrograde Evan's blue staining; red and white, area at risk; white, infarcted tissue (right; scale bar, 50 μm). $*P < 0.05$. $n = 8$ mice per group. Statistical significance was calculated by one-way ANOVA with Dunnett's *post-hoc* test. Data are mean ± s.d. (**g**) Schematic model of Adora2b-dependent Per2 stabilization and its role in regulating glycolysis and cardiac metabolism during myocardial ischemia. pCREB, phosphorylated CREB.

daylight for 4 h induced cardiac glycolytic enzyme mRNA expression in wild-type but not *Per2*$^{-/-}$ mice (**Fig. 6c**).

We next tested whether light-exposure–elicited stabilization of Per2 could lead to cardioprotection. We observed a time-dependent attenuation of myocardial infarct sizes and plasma troponin I concentrations in wild-type mice pre-exposed to illumination with an equivalent intensity to that of daylight (13,000 lx), but not in *Per2*$^{-/-}$ mice (**Fig. 6d**,**e** and **Supplementary Fig. 19b**). Consistent with these findings, myocardial infarct size and troponin I concentrations in serum samples from wild-type mice showed diurnal variations, with significantly smaller infarct sizes and plasma troponin I concentrations at ZT12 and ZT18 compared to ZT0 (**Fig. 6f** and **Supplementary Fig. 19c**). Taken together, these findings indicate that exposure to intense light enhances cardiac Per2 concentrations and is associated with Per2-dependent cardioprotection from ischemia (**Fig. 6g**).

## DISCUSSION

Myocardial adaptation to conditions of limited oxygen availability involves a metabolic switch toward a more oxygen-efficient utilization of carbohydrates[27]. Our results show that ADORA2B-dependent stabilization of PER2 has a key role in this cardioadaptive response. We found that *Per2*$^{-/-}$ mice have diminished amounts of cellular energy stores during ischemia and did not generate lactate. *Per2*$^{-/-}$ mice lacked the capacity to enhance oxygen-efficient glycolysis, ultimately resulting in the depletion of energy-rich phosphates and leading to increased myocardial cell death during ischemia. Together, these studies indicate a previously unrecognized role for Per2 as a master metabolic switch during cardioadaption to ischemia, driving the utilization of oxygen-efficient carbohydrate-dependent metabolic pathways (**Fig. 6g**).

We found that Adora2b signaling increased Per2 expression and protein stability. A previous study showed that Adora2b signaling activates mitogen-activated protein kinases (MAPK) through the cAMP-CREB pathway[28], which have been implicated in Per2 regulation[29]. Consistent with the notion that Adora2b signaling alters intracellular cAMP responses, recent studies have identified CLOCK protein-independent regulation of Per2 by cAMP-dependent signaling[30]. Other studies have shown that Adora2b signaling regulates the stability of target proteins. For example, study of hypoxic preconditioning of the lungs showed that Adora2b signaling has protective and anti-inflammatory effects by regulating the post-translational stability of NF-κB[15].

Our studies show that cardiac Per2 stabilization can be achieved by daylight exposure. Indeed, stabilization of Per2 within the suprachiasmatic nuclei of the hypothalamus involves light-induced Creb phosphorylation[31]. Moreover, previous studies found that the timing of light exposure markedly affects the circadian profiles of Per1 or Per2 protein concentrations in the lungs and hearts of rats[26,32]. The mechanisms linking central circadian rhythm regulation and peripheral Per2 stabilization are currently under investigation and may involve hormonal pathways[33] or cyclic alterations in 5′-AMP or adenosine concentrations[34]. Indeed, a recent study identified 5′-AMP in the initiation of hypometabolism, similar to a torpor-like state found in hibernating mammals[35].

It is intriguing to consider the findings of light-dependent stabilization of Per2 and cardioprotection from ischemia in the context of well-documented variations in the frequency of the onset of acute myocardial infarction[36]. Two recent studies found that patients have larger infarct sizes in the early morning hours compared to other times of the day[37,38]; moreover, myocardial infarct size and left ventricular function after acute myocardial infarction have a circadian dependence on the time of day of the onset of ischemia[38]. However, another study reported a 12-h delay in the oscillation of cardiac PER2 concentrations in humans as compared to mice[39]. In this respect, observations in patients indicating larger infarct sizes in the morning hours[37] are in contrast to our present findings. Notably, the study characterizing the oscillation of cardiac PER2 using human tissue obtained during orthotopic heart transplantation (ref. 39) included a high proportion of patients with coronary heart disease and cardiomyopathy compared to healthy controls (patients without cardiac disease who died from head injury). Therefore, it is not clear whether the PER2 expression pattern observed is characteristic of hearts in normal, healthy individuals, and this may help explain the apparent difference in the circadian pattern of PER2 expression between humans and mice. Moreover, epidemiologic studies in humans have indicated that sunlight is the dominant zeitgeber (a signal from the environment that synchronizes the human circadian system) compared to other zeitgebers such as social cues. This would indicate that the oscillation of cardiac Per2 could be very similar in humans as in mice, as both mice and humans are exposed to the same day-night rhythm[40]. However, the present findings in mice cannot simply be extrapolated to the circadian rhythmicity of myocardial ischemia in humans. Additional studies will be necessary to define the expression and circadian rhythmicity of PER2 in the human heart, as well as its functional role in human heart disease.

The key role of anaerobic glycolysis in providing ATP in severe ischemia has been well documented in mice. After low-flow myocardial ischemia, mice deficient in the glucose transporter Glut4 (ref. 41) or in AMP kinase[42] have reduced lactate production, associated with increased tissue injury, and upon reperfusion have diminished regeneration of high-energy phosphate compounds. These findings are in line with our studies showing larger infarct sizes in *Per2*$^{-/-}$ mice, which did not utilize glycolysis during ischemia or restore glycogen concentrations during reperfusion. Similarly to Glut4-deficient mice, *Per2*$^{-/-}$ mice showed higher glycogen concentrations at baseline but were unable to sufficiently utilize carbohydrates. Although diminished uptake of exogenous glucose contributes to the phenotype of Glut4-deficient mice, we did not observe any differences in $^{13}$C-6-glucose uptake between wild-type and *Per2*$^{-/-}$ mice.

Our results indicate that Hif-1α regulates the Per2-dependent enhancement of glycolytic capacity during ischemia. These findings are consistent with recent studies of myocardial ischemia in mice that are hypomorphic for HIF prolyl 4-hydroxylase-2 (ref. 43). These mice have increased protein concentrations of Hif-1α and Hif-2α in conjunction with cardioprotection from ischemia. Consistent with our results, which show that *Per2*$^{-/-}$ mice have decreased Hif-1α expression, larger infarct sizes and attenuated glycolytic flow, that study[43] showed that Hif-1α overexpression is associated with increased lactate concentrations and increased glycolytic capacity[43]. Although regulation of the expression of the glycolytic enzyme Ldh by Hif-1α has been previously documented[44], the Clock:Bmal1 dimer transcription factor rather than Hif-1α has been implicated in regulating cardiac Ldh expression[45]. Clock:Bmal1 has also been shown to regulate the circadian pattern of Per2 expression[13]. However, photic induction of Per2 in the suprachiasmatic nuclei has been suggested to be Creb-dependent[46]. Similarly, we found that enhanced Adora2b signaling led to the induction of Creb activity and Per2 stabilization. Taken together, previous findings and our current work support the concept that multiple pathways can function to regulate the circadian network[47].

Consistent with our findings that cardiac Hif-1α has a circadian pattern of expression, at highest concentrations in the late evening (ZT12–ZT18), we found a significant reduction in infarct sizes at ZT12 and ZT18 compared to ZT0. However, another study on diurnal variations in myocardial ischemia and reperfusion tolerance in mice found an increased infarct size at ZT12 compared to ZT0 (ref. 48). These contrasting results might be due to differing experimental conditions. Whereas the other study[48] used a closed chest model for myocardial ischemia and a potent opioid (buprenorphine) for anesthesia, we used an open chest model, which has been associated with increased inflammation[49], and which may have influenced our experimental results. Moreover, in contrast to our results, a previous study[50] showed that *Per2*$^{-/-}$ mice have attenuated infarct sizes after permanent cardiac occlusion; these differing results could be due to the difference in the type of cardiac injury studied or to the methods used (for example, in measuring the area at risk). In line with our findings, other studies on the role of *Per2* during ischemia and reperfusion have reported impaired endothelial progenitor cell function and autoamputation of the distal limb when *Per2*$^{-/-}$ mice were subjected to hind-limb ischemia[51,52], indicating that Per2 is crucial in promoting ischemia tolerance. Unlike Per2 deficiency, Per1 deficiency did not affect the response to myocardial ischemia or HIF-1α expression, consistent with previous studies showing nonredundant roles for Per1 and Per2 in the mammalian circadian clock[53].

A limitation of our work is the use of a germline *Per2*$^{-/-}$ mouse, which does not allow us to make conclusions regarding the contributions of different tissues to the observed phenotype. Therefore, additional studies will need to include mice with tissue-specific *Per2* deletion.

Our results identify Adora2b-dependent stabilization of Per2 as an endogenous mechanism that allows the ischemic myocardium to adapt its metabolism toward the oxygen-efficient utilization of carbohydrates. Future challenges will involve understanding the regulatory axis by which light exposure leads to cardiac Per2 stabilization, as well as defining approaches to stabilize cardiac Per2 concentrations in a therapeutic setting.

## METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

*Note: Supplementary information is available on the Nature Medicine website.*

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## AUTHOR CONTRIBUTIONS

T.E. designed and supervised the study, wrote the manuscript and did mouse surgery. K.H. did western blots, RT-PCRs and siRNA knockdown studies. S.B. did western blots, coimmunoprecipitation, promoter studies, ELISAs and animal experiments. S.R. did western blots, RT-PCRs, ELISAs and mouse experiments. M.M. did immunohistochemistry and electron microscopy. L.A.W. isolated mouse myocytes and supervised the study. B.D.L. provided human heart samples. J.H., C.H.B. and D.J.K. did metabolic analysis. P.M.B., S.P.C. and H.K.E. supervised the study and wrote the manuscript.

## COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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# Nuclear accumulation of HDAC4 in ATM deficiency promotes neurodegeneration in ataxia telangiectasia

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**Ataxia telangiectasia is a neurodegenerative disease caused by mutation of the *Atm* gene. Here we report that ataxia telangiectasia mutated (ATM) deficiency causes nuclear accumulation of histone deacetylase 4 (HDAC4) in neurons and promotes neurodegeneration. Nuclear HDAC4 binds to chromatin, as well as to myocyte enhancer factor 2A (MEF2A) and cAMP-responsive element binding protein (CREB), leading to histone deacetylation and altered neuronal gene expression. Blocking either HDAC4 activity or its nuclear accumulation blunts these neurodegenerative changes and rescues several behavioral abnormalities of ATM-deficient mice. Full rescue of the neurodegeneration, however, also requires the presence of HDAC4 in the cytoplasm, suggesting that the ataxia telangiectasia phenotype results both from a loss of cytoplasmic HDAC4 as well as its nuclear accumulation. To remain cytoplasmic, HDAC4 must be phosphorylated. The activity of the HDAC4 phosphatase, protein phosphatase 2A (PP2A), is downregulated by ATM-mediated phosphorylation. In ATM deficiency, enhanced PP2A activity leads to HDAC4 dephosphorylation and the nuclear accumulation of HDAC4. Our results define a crucial role of the cellular localization of HDAC4 in the events leading to ataxia telangiectasia neurodegeneration.**

Ataxia telangiectasia is a hereditary multisystemic disease resulting from mutations in the *ATM* gene, which encodes a 370-kD member of the phosphatidylinositol 3 (PI3)-kinase family of kinases[1–4]. The symptoms of ataxia telangiectasia include non-neurological phenotypes such as immune-system defects, germ-cell defects, hypersensitivity to ionizing radiation and increased susceptibility to cancer[2,3,5]. However, it is the loss of neuronal cells, the most direct cause of the devastating ataxia, that is arguably the least understood phenotype in this disease. We report a newly discovered role for HDAC4 in this process.

Class I and class IIa histone deacetylases (HDACs) have key roles in brain development and neuron survival[6–10]. HDAC4 (class IIa) is abundant in neurons, where it is predominantly cytoplasmic[11–13]. Relevant to the phenotype of ataxia telangiectasia, HDAC4 deficiency in mice is marked by a postnatal atrophy of the cerebellum, with surviving Purkinje cells having a notable reduction in dendritic complexity[14]. HDAC4 is normally phosphorylated by calcium/calmodulin-dependent kinases (CaMKs), enabling its binding to the 14-3-3 family of protein chaperones[7,15–17]. HDAC4, like other class IIa HDACs, associates with the prosurvival transcription factors MEF2A and CREB, repressing their transcriptional activity[18–20]. Though well studied in other cell types, the relationship between HDAC4 and MEF2A- or CREB-dependent gene expression in neurons is largely unexplored.

## RESULTS

### Nuclear HDAC4 reduces MEF2A/CREB-dependent transcription

The similar phenotypes of neuron death and dendritic atrophy in the cerebella of ATM- or HDAC4-deficient mice[9,18,21,22] prompted us to examine HDAC4 in human ataxia telangiectasia cerebella. Normally, HDAC4 immunoreactivity is found in Purkinje cell cytoplasm (**Fig. 1a**). However, the Purkinje cell nuclei in the ataxia telangiectasia samples had strong HDAC4 staining (**Fig. 1a**,**b**). The nuclear accumulation of HDAC4 was specific: despite structural and functional similarities to HDAC4 (refs. 23,24), HDAC5 and HDAC9 showed little nuclear accumulation in ataxia telangiectasia (**Supplementary Fig. 1a**). We replicated these observations in mice. HDAC4, but not HDAC5 or HDAC9 (**Supplementary Fig. 1b**), showed significant nuclear accumulation in $Atm^{-/-}$ but not wild-type mouse Purkinje cells (**Fig. 1a**,**c**). We found similar shifts in HDAC4 localization in other mouse brain regions, including the neocortex and brain stem (**Supplementary Fig. 1c**). The shift was also seen with exogenous protein; GFP-HDAC4 transfected into cultured $Atm^{-/-}$ neurons also localized to the nucleus (**Fig. 1d**). Further, after exposure of cultured cortical neurons to siRNA against *Atm*, HDAC9 remained in the cytoplasm, whereas endogenous HDAC4 shifted to the nucleus (**Supplementary Fig. 1d**).

The association of MEF2A and CREB with HDAC4 was markedly higher in $Atm^{-/-}$ cerebellar extracts than in wild-type extracts (**Fig. 1e**,**f** and **Supplementary Fig. 2a**). That interaction with HDAC4 served to inactivate MEF2A and CREB because we observed reduced promoter occupancy of these two proteins at known target genes in $Atm^{-/-}$ mice. We performed chromatin immunoprecipitation (ChIP) and assayed by quantitative PCR (qPCR) the extent to which various promoter regions were occupied: *Egr3*, *Fos* and *Mef2a* for MEF2A and *Atf*, *Nrxn1*, *Nrxn3* and *Egr1* for CREB. In each region, we found lower

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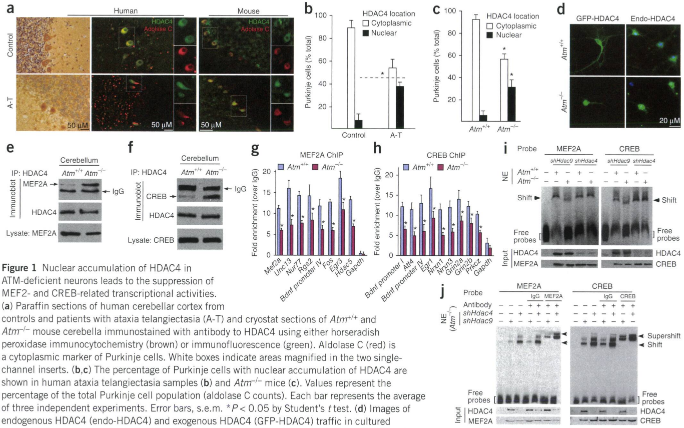
a
Human
Mouse
Control
A-T
HDAC4
Aldolase C
50 μM
b
HDAC4 location
Cytoplasmic
Nuclear
Purkinje cells (% total)
Control
A-T
c
HDAC4 location
Cytoplasmic
Nuclear
Purkinje cells (% total)
Atm+/+
Atm−/−
d
GFP-HDAC4
Endo-HDAC4
Atm+/+
Atm−/−
20 μM
e
Cerebellum
IP: HDAC4
Atm+/+ Atm−/−
Immunoblot
MEF2A
IgG
HDAC4
Lysate: MEF2A
f
Cerebellum
IP: HDAC4
Atm+/+ Atm−/−
Immunoblot
CREB
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Lysate: CREB
g
MEF2A ChIP
Atm+/+ Atm−/−
Fold enrichment (over IgG)
Mef2a
Unc13
Nur77
Rgs2
Bdnf promoter IV
Fos
Egr3
Hdac5
Gapdh
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CREB ChIP
Atm+/+ Atm−/−
Fold enrichment (over IgG)
Bdnf promoter I
Atf4
Bdnf promoter IV
Egr1
Nrxn1
Nrxn3
Grin2a
Grin2b
Prkcz
Gapdh
i
Probe
MEF2A
CREB
shHdac9 shHdac4
NE
Atm+/+
Atm−/−
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Antibody
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shHdac9
Supershift
Shift
Free probes
Input
HDAC4
MEF2A
CREB

**Figure 1** Nuclear accumulation of HDAC4 in ATM-deficient neurons leads to the suppression of MEF2- and CREB-related transcriptional activities. (**a**) Paraffin sections of human cerebellar cortex from controls and patients with ataxia telangiectasia (A-T) and cryostat sections of $Atm^{+/+}$ and $Atm^{-/-}$ mouse cerebella immunostained with antibody to HDAC4 using either horseradish peroxidase immunocytochemistry (brown) or immunofluorescence (green). Aldolase C (red) is a cytoplasmic marker of Purkinje cells. White boxes indicate areas magnified in the two single-channel inserts. (**b**,**c**) The percentage of Purkinje cells with nuclear accumulation of HDAC4 are shown in human ataxia telangiectasia samples (**b**) and $Atm^{-/-}$ mice (**c**). Values represent the percentage of the total Purkinje cell population (aldolase C counts). Each bar represents the average of three independent experiments. Error bars, s.e.m. $*P < 0.05$ by Student's *t* test. (**d**) Images of endogenous HDAC4 (endo-HDAC4) and exogenous HDAC4 (GFP-HDAC4) traffic in cultured neocortical neurons from both $Atm^{+/+}$ and $Atm^{-/-}$ embryos. (**e**) Protein extracts from $Atm^{+/+}$ and $Atm^{-/-}$ mouse cerebella immunoprecipitated (IP) with HDAC4 and blotted with antibodies to MEF2A. IgG, immunoglobulin G. (**f**) Protein extracts from $Atm^{+/+}$ and $Atm^{-/-}$ mouse cerebella immunoprecipitated with HDAC4 and blotted with antibodies to CREB. (**g**) After ChIP with antibodies to MEF2A from $Atm^{+/+}$ and $Atm^{-/-}$ cerebela, a quantitative real-time PCR analysis was performed for the presence of specific MEF2A target genes. (**h**) After ChIP with antibodies to CREB from $Atm^{+/+}$ and $Atm^{-/-}$ cerebella, a quantitative real-time PCR analysis was performed for the presence of specific CREB target genes. $*P < 0.01$ by Student's *t* test (**f**,**h**). *Gapdh* was used as a control. Error bars, s.e.m. All the qPCR primers used are listed in **Supplementary Table 2**. (**i**,**j**) Validation of the effect of nuclear HDAC4 on the MEF2A-DNA and CREB-DNA interactions in $Atm^{-/-}$ neurons. Nuclear extracts (NE) from $Atm^{+/+}$ and $Atm^{-/-}$ neurons with lentiviral *Hdac9* shRNA (*shHdac9*) and *Hdac4* shRNA (*shHdac4*) infection were incubated with biotin-labeled probes, as indicated.

promoter occupancy in the $Atm^{-/-}$ samples compared to the wild-type samples (**Fig. 1g,h**). We next performed electrophoretic mobility shift assays with MEF2A and CREB DNA-binding consensus sequences (**Supplementary Table 1**). Nuclear extracts from $Atm^{-/-}$ mouse brains showed lower binding to these probes compared to wild-type brains (**Fig. 1i**). Further, the DNA binding of MEF2A and CREB, as well as their supershifts, could be restored in $Atm^{-/-}$ cells by knocking down HDAC4 but not HDAC9 (**Fig. 1i,j**). We determined that the probes were specific (**Supplementary Fig. 2b**), and the shRNAs used for the knockdown were efficacious (**Supplementary Fig. 2c**).

## Nuclear HDAC4 suppresses neuronal gene expression

Enhanced HDAC4 in $Atm^{-/-}$ neuronal nuclei suggests a reduction of histone acetylation in these cells. Immunostaining for total histone 3 (H3) and H4 in $Atm^{-/-}$ cerebella was similar to that in wild-type cerebella (**Fig. 2a**). By contrast, we found less immunostaining for acetylated histone 3 (AcH3) and AcH4 in $Atm^{-/-}$ mice compared to wild-type mice. We found the same result in the neocortex and hippocampus (**Supplementary Fig. 2d**). We confirmed these findings using western blots (**Fig. 2b–d**). We then examined the association of AcH3 and AcH4 with specific gene promoters. We performed ChIP followed by qPCR assays with extracts from wild-type and $Atm^{-/-}$ mouse cerebella (**Fig. 2e–g**). The ChIP of H3 revealed no consistent difference between wild-type and $Atm^{-/-}$ samples in chromatin association at the promoters we examined (**Fig. 2e**). By contrast, the associations of AcH3 and AcH4 with multiple neuronal growth genes were consistently lower in $Atm^{-/-}$ samples compared to wild-type samples. The loss of histone acetylation in a promoter region suggests a closed chromatin configuration and reduced transcription. Accordingly, in $Atm^{-/-}$ compared to wild-type samples, we observed less transcription of multiple neuronal genes at which there was lower promoter occupancy by acetylated histones, for example, *Bdnf*, *Grin2a* (also known as *Nr2a*), *Nrxn1* and *Nrxn3* (**Fig. 2f–g**). The occupancy of these promoters by H3 (**Fig. 2e**) and H4 (data not shown) in $Atm^{-/-}$ mice was equivalent to that in wild-type mice.

We next performed ChIP with HDAC4 and found that it directly associates with chromatin (**Supplementary Fig. 3a,b**). We analyzed the HDAC4-precipitated DNA with ChIP sequencing (ChIP-seq) using a SOLiD DNA fragment library platform. The aligned ChIP-seq tags revealed patterns of HDAC4-chromatin binding that differed substantially between wild-type and $Atm^{-/-}$ mice (**Fig. 2h** and **Supplementary Fig. 3c**). To validate the ChIP-seq results, we performed ChIP-qPCR of selected genes using primers designed to be within the ChIP-seq–identified $Atm^{-/-}$ peak, as well as primers in two sites flanking the peak by approximately 900 bp (**Supplementary Fig. 3d–g**). All four qPCR assays showed

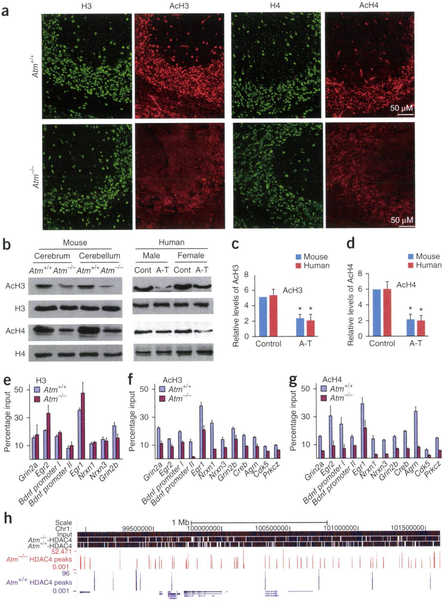

**Figure 2** Nuclear accumulation of HDAC4 leads to global effects on histone acetylation and neuronal gene expression. (**a**) Fluorescent images of sections from *Atm*$^{+/+}$ and *Atm*$^{-/-}$ mouse cerebella immunostained for two histones (H3 and H4) and the acetylated histones (AcH3 and AcH4), as indicated. (**b**) Protein extracts of cortices and cerebella from wild-type and *Atm*$^{-/-}$ mice probed with antibodies to various histones, with the histones labeled to the left of the gels. Cont, control. (**c**,**d**) Quantification of three repetitions of the experiment illustrated in panel **b**. Error bars, s.d. $*P < 0.05$ by Student's *t* test. (**e**–**g**) Fragmented chromatin immunoprecipitated with antibodies to H3, AcH3 or AcH4, as indicated, and quantified using real-time PCR. The primers used for the qPCR are listed in **Supplementary Table 2**. Error bars, s.e.m. (**h**) An illustration of the HDAC4 ChIP-seq alignment and peaks. A 2.7-Mb sample region of chromosome 1 (Chr1) shows the density of coverage of 35-nt sequencing tags from input DNA or ChIP from wild-type (blue) or *Atm*$^{-/-}$ (red) mouse brain. WT-HDAC4, wild-type HDAC4; *Atm*$^{-/-}$-HDAC4, HDAC4 in *Atm*$^{-/-}$ (knockout) mice.

increased HDAC4 binding to the region of the peak predicted by sequencing and peak finding in the *Atm*$^{-/-}$ samples.

### Inhibiting HDAC4 prevents neuronal dysfunction in *Atm*$^{-/-}$

Our data indicate that shifting the location of HDAC4 from the cytoplasm to the nucleus is neurotoxic. Notably, HDAC4 knockout mice have smaller brains than wild-type mice, suggesting that genetic deletion of HDAC4 is also neurotoxic[14]. To explore this potential discrepancy, we treated *Atm*$^{-/-}$ and wild-type mice with trichostatin A (TSA), a classic HDAC inhibitor[25–27]. We used treatment with sodium butyrate, a class I HDAC inhibitor, as a control. After 7 d of TSA treatment, the concentrations of markers of degeneration, such as cleaved caspase-3 and the cell cycle markers proliferating cell nuclear antigen (PCNA) and cyclin D1, were substantially lower than those normally found in *Atm*$^{-/-}$ cerebellum (**Fig. 3a**,**b**); by contrast, the concentrations of neuronal survival proteins were higher (**Fig. 3c**,**d**). Treatment with sodium butyrate had little effect on the levels of these markers (**Supplementary Fig. 4a**,**b**).

*Atm*$^{-/-}$ mice have motor abnormalities that are consistent with cerebellar dysfunction[28], and our data (**Fig. 3a–d**) predicted that treatment with TSA should prevent these abnormalities. We treated wild-type and *Atm*$^{-/-}$ mice for 3 weeks with either TSA or sodium butyrate. We then trained the mice for 1 week on a rotating rod (rotarod) and tested their motor performance. Both genotypes performed equally well on the rotarod at slow rotation speeds (10 rpm; data not shown). At more strenuous speeds (16 rpm), the performance of the *Atm*$^{-/-}$ mice was significantly worse than the wild-type mice; after TSA treatment, however, the mutant mice performed similarly to the treated wild-type mice ($P < 0.05$; **Fig. 3e**). We saw a similar effect on spontaneous locomotor activity (**Fig. 3f**), as measured by open-field behavior. The exploratory activity (rearing; **Fig. 3g**) of TSA-treated *Atm*$^{-/-}$ mice was significantly greater than that of untreated *Atm*$^{-/-}$ mice ($P < 0.05$) and approached the values found for wild-type mice. Treatment with sodium butyrate had little effect on the performance of *Atm*$^{-/-}$ mice in any behavioral tests (**Fig. 3e–g**).

### Hypophosphorylation of HDAC4 induces its nuclear accumulation

To determine whether HDAC4 has a role in the neuronal DNA damage response, we exposed cultures of wild-type and *Atm*$^{-/-}$neurons to low doses of etoposide, a topoisomerase inhibitor known to induce DNA double-strand breaks. This exposure enhanced the activation of caspase-3, but this effect was tenfold greater in *Atm*$^{-/-}$ neurons than in wild-type neurons (**Fig. 4a**,**b**). When we used shRNA against HDAC4 in addition to (and at the same time as) the etoposide exposure, however, the cell death induced by etoposide increased in the wild-type neurons, but the shRNA had little effect on cell death in the *Atm*$^{-/-}$ neurons (**Fig. 4a**,**b**). Thus, loss of cytoplasmic HDAC4

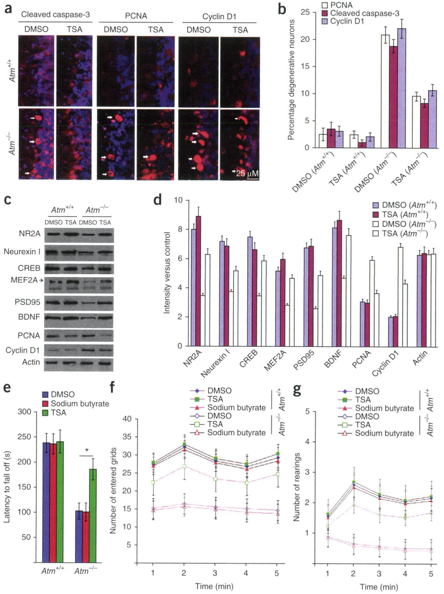

**Figure 3** Inhibition of HDAC4 and blocking the nuclear accumulation of HDAC4 partially reverses the ataxia telangiectasia phenotype. (**a**) TSA injection reverses neuronal degeneration markers in the $Atm^{-/-}$ cerebellum. Fluorescent images of $Atm^{-/-}$ brain sections immunostained for cleaved caspase-3, as well as for PCNA and cyclin D1. The white arrows indicate labeled Purkinje cells. (**b**) Quantification of the degeneration markers for the experiment shown in **a**. Each bar represents the average of three independent experiments. Error bars, s.e.m. (**c**) Immunoblot assays of neuronal and cell-cycle proteins in cerebellar lysates prepared from DMSO- or TSA-injected wild-type and $Atm^{-/-}$ mice. (**d**) Quantification of the western blot bands shown in **c**. Error bars, s.d. (**e**) Effects of TSA on the motor function of $Atm^{-/-}$ and wild-type mice. Motor performance was measured as the average latency before falling from a rotarod. Each treatment group consisted of 4–6 mice. $^*P < 0.05$ by analysis of variance (ANOVA). (**f**,**g**) Effects of TSA on the spontaneous locomotor activities (**f**) and the exploratory activities (**g**) in $Atm^{-/-}$ mice, as observed by open-field test. Data are means ± s.e.m.

makes neurons more sensitive to DNA damage. However, HDAC4 itself does not respond to DNA damage. We exposed wild-type and $Atm^{-/-}$ mice to 5 Gy of whole-body irradiation, which activated ATM in wild-type mice but not in $Atm^{-/-}$ mutant mice (**Fig. 4c**). The cytoplasmic location of HDAC4 nonetheless was unchanged after irradiation in mice of both genotypes (**Fig. 4c**).

We prepared cytoplasmic and nuclear fractions from wild-type and $Atm^{-/-}$ mouse cerebella and probed them with an antibody against phosphorylated Ser632 (phospho-Ser632) of HDAC4 or total HDAC4. In the wild-type cerebellum, most of the HDAC4 was cytoplasmic and phosphorylated (**Fig. 4d**,**e**). In the $Atm^{-/-}$ cerebellum, however, a large portion of the HDAC4 was nuclear and was almost entirely unphosphorylated at that location. The total HDAC4 was the same in wild-type and $Atm^{-/-}$ mice (**Fig. 4d**,**e**). Homogenates of human ataxia telangiectasia cerebellar samples behaved similarly to the $Atm^{-/-}$ mouse samples (**Fig. 4f**), and the use of a second HDAC4 phosphospecific antibody (to phospho-Ser246) confirmed the phospho-Ser632 results (**Fig. 4f**). The lower amount of HDAC4 phosphorylation in $Atm^{-/-}$ mice compared to wild-type mice led to a lower association of HDAC4 with the 14-3-3 protein, as assayed by coimmunoprecipitation from human ataxia telangiectasia cerebellum (**Fig. 4g**,**h**) and $Atm^{-/-}$ mouse brain (**Supplementary Fig. 5a**,**b**).

We could find no canonical Ser/Thr-Gln ATM target site on HDAC4 itself or on the kinases responsible for its phosphorylation, CaMKII and CaMKIV[7,17,29]. Therefore, we asked whether the HDAC4 protein phosphatase, PP2A[30], was involved in the hypophosphorylation of HDAC4 in $Atm^{-/-}$ mice. PP2A is a heterotrimeric protein[31] that must associate with its enzymatic target for full phosphatase activity. It consists of a structural A subunit (PP2A-A, also known as PR65), a tissue-specificity–regulatory B subunit (PP2A-B, also known as PR55) and a catalytic C subunit (PP2A-C, also known as Pp2ca). Immunoprecipitations of HDAC4 from human control lysates revealed little interaction of HDAC4 with either the PP2A-A or PP2A-C subunit. By contrast, in the patients with ataxia telangiectasia, we observed a robust association of HDAC4 with both the A and C subunits of PP2A (**Fig. 4i**). We also found this greater HDAC4-PP2A association in the $Atm^{-/-}$ mouse brain (**Supplementary Fig. 5c**). Thus, the lower phosphorylation of HDAC4 in ATM-deficiency is likely caused by a greater association of HDAC4 with its phosphatase, PP2A.

## PP2A mediates ATM-dependent HDAC4 nuclear translocation

Ser401 of PP2A-A is a highly probable site of ATM phosphorylation[32]. We therefore performed immunoprecipitation with antibodies to PP2A-A, PP2A-C or HDAC4 and probed the resulting western blots with an antibody to phosphorylated serine or threonine preceding a glutamine residue (phospho-Ser/Thr-Gln), the canonical ATM and ataxia telangiectasia and Rad3 related (ATR) target binding site.

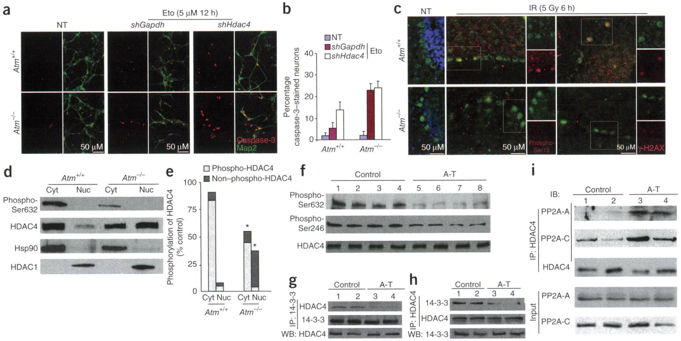

**Figure 4** HDAC4 cytoplasmic localization requires phosphorylation of HDAC4 and is independent of DNA damage. (**a**) The effect of *Hdac4* shRNA on caspase-3 activation in $Atm^{-/-}$ neurons. Activation of caspase-3 (red) was used as an index of impending neurodegeneration; microtubule-associated protein 2 (Map2) (green) was used as a neuronal marker. Eto, etoposide; NT, no treatment; *shGapdh*, *Gapdh* shRNA. (**b**) Cell death quantified by counting the number of activated caspase-3–immunostained cells and expressing these numbers as a percentage of the total Map2-stained neurons. Data are mean ± s.e.m. (**c**) Mice treated with or without 5 Gy whole-body irradiation (IR). Cryostat sections of $Atm^{+/+}$ and $Atm^{-/-}$ cerebella immunostained for HDAC4 (green) and H2A histone family member X (γ–H2AX) or phospho-Ser15 of p53 (both in red). At least three pairs of age-matched mice were used for each experiment. White boxes indicate areas magnified in the two single-channel insets. (**d**) Immunoblot assays of HDAC4 and phospho-S632 of HDAC4 in nuclear (Nuc) or cytoplasmic (Cyt) extracts prepared from $Atm^{+/+}$ and $Atm^{-/-}$ mouse cerebella. Heat shock protein 90 (Hsp90) and HDAC1 were used as cytoplasmic and nuclear markers, respectively. (**e**) A quantification of the bands shown in **d**. $^{*}P < 0.05$ by Student's *t* test. (**f**) Immunoblot assays of HDAC4 and phospho-HDAC4 in protein extracts prepared from frozen cerebellar samples from four human controls without ataxia telangiectasia and four individuals with ataxia telangiectasia. (**g**,**h**) Coimmunoprecipitations showing the interaction between HDAC4 and 14-3-3 protein in lysates of cerebellar tissue from human control and ataxia telangiectasia brains. WB, western blot. (**i**) Coimmunoprecipitations showing the association of HDAC4 with the PP2A subunits in lysates of cerebellar tissue from human control and ataxia telangiectasia brain. IB, immunoblot.

We found a strong phospho-Ser/Thr-Gln signal on the PP2A-A band from wild-type but not $Atm^{-/-}$ mice on the western blot (**Fig. 5a**). We found no phospho-Ser/Thr-Gln signal for either wild-type or $Atm^{-/-}$ mice with the PP2A-C or HDAC4 immunoprecipitates (**Fig. 5a**). As further proof that HDAC4 itself is not an ATM substrate, we also found no phospho-Ser/Thr-Gln signal in N2a cells overexpressing Flag-tagged HDAC4 (Flag-HDAC4) (**Supplementary Fig. 6a**). We verified that Ser401 is the predominant ATM phosphorylation site on PP2A using *in vitro* kinase assays (**Fig. 5b**); a form of PP2A-A carrying the S401A mutation could not be phosphorylated by ATM. We overexpressed Flag-HDAC4 with GFP-tagged isoforms of PP2A-A and analyzed the Flag-HDAC4 immunoprecipitates for the presence of PP2A-A. We found a strong HDAC4-PP2A association in the nonphosphorylatable (S401A) PP2A-A isoform (S401A) (**Fig. 5c**) but not in wild type or in the phosphomimetic isoform (S401D) (**Fig. 5c**). We confirmed this result by probing PP2A-A immunoprecipitates for Flag-HDAC4 (**Fig. 5c**).

ATM-dependent phosphorylation of PP2A-A also alters the localization of the PP2A holoenzyme itself. Endogenous PP2A-A was predominantly cytoplasmic in wild-type neurons but was predominantly nuclear in $Atm^{-/-}$ neurons (**Fig. 5d**). In wild-type primary neurons, when we coexpressed mutant or wild-type GFP–PP2A-A with mCherry-tagged PP2A-C (mCherry–PP2A-C), both subunits were located primarily in the cytoplasm (**Fig. 5e**). In $Atm^{-/-}$ neurons, however, unmodified PP2A-A and PP2A-C translocated to the nucleus, whereas the S401D PP2A-A mutant was located predominantly in the cytoplasm, even in ATM-deficient neurons. The S401A PP2A-A mutant was nuclear in both wild-type and $Atm^{-/-}$ neurons (**Fig. 5e**). In contrast, the locations of the HDAC4 kinases CaMKII and CaMKIV were unaffected by ATM deficiency (data not shown).

We inhibited ATM activity with 2 mM caffeine or 10 µM KU-55933. Both inhibitors caused HDAC4 to move from cytoplasm to nucleus within 3 h of administration (**Fig. 5f** and **Supplementary Fig. 6b**). This translocation of HDAC4 could be blocked by simultaneously knocking down PP2A (**Fig. 5g** and **Supplementary Fig. 6c**) or by pretreating the cells with the PP2A inhibitor endothall (**Fig. 5h** and **Supplementary Fig. 6d**). The genotype-dependent nuclear translocation of endogenous HDAC4 could also be blocked by infecting $Atm^{-/-}$ neurons with *Pr65* (also known as *Ppp2r1a*) or *Pp2ca* shRNAs (**Fig. 5h**).

## Cytoplasmic HDAC4 improves the phenotype of $Atm^{-/-}$

To verify that cytoplasmic HDAC4 prevents cell cycle reentry and other degenerative changes in $Atm^{-/-}$ mice, we co-injected lentiviral particles encoding human wild-type HDAC4, a nuclear localization HDAC4 mutant (4A, which has the R269A, R280A, K280A and R281A mutations), a nuclear export mutant (L1062A) or a nonphosphorylatable mutant (3SA, which has the S246A, S467A and

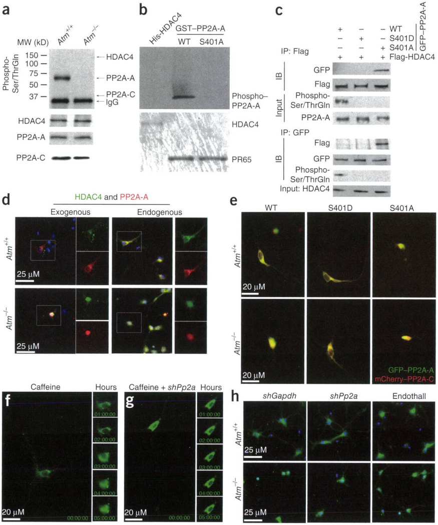
a
MW (kD)
Atm+/+
Atm−/−
Phospho-Ser/ThrGln
150 —
100 —
75 —
50 —
37 —
HDAC4
PP2A-A
PP2A-C
IgG
HDAC4
PP2A-A
PP2A-C
b
His-HDAC4
GST–PP2A-A
WT
S401A
Phospho–PP2A-A
HDAC4
PR65
c
+ − − WT
− + − S401D
− − + S401A
GFP–PP2A-A
+ + + Flag-HDAC4
IP: Flag
IB
GFP
Flag
Input
Phospho-Ser/ThrGln
PP2A-A
IP: GFP
IB
Flag
GFP
Phospho-Ser/ThrGln
Input: HDAC4
d
HDAC4 and PP2A-A
Exogenous
Endogenous
Atm+/+
Atm−/−
25 μM
25 μM
e
WT
S401D
S401A
Atm+/+
Atm−/−
20 μM
20 μM
GFP–PP2A-A
mCherry–PP2A-C
f
Caffeine
Hours
20 μM
00:00:00
01:00:00
02:00:00
03:00:00
04:00:00
05:00:00
g
Caffeine + shPp2a
Hours
20 μM
00:00:00
01:00:00
02:00:00
03:00:00
04:00:00
05:00:00
h
shGapdh
shPp2a
Endothall
Atm+/+
Atm−/−
25 μM
25 μM

**Figure 5** The PP2A-A subunit, PR65, is a ATM target and mediates nuclear accumulation of HDAC4 in ATM-deficient neurons. (**a**) Protein extracts from *Atm*$^{+/+}$ and *Atm*$^{-/-}$ mouse cerebella immunoprecipitated with PP2A-A, PP2A-C or HDAC4 and blotted with antibodies to phospho-Ser/Thr-Gln. MW, molecular weight. (**b**) *In vitro* ATM kinase assays of His-tagged HDAC4 (His-HDAC4), GST-tagged PP2A-A (GST–PP2A-A) or PP2A-A with the S401A mutation performed with N2a cell extract. (**c**) Coimmunoprecipitation assays of PP2A-A and HDAC4 in lysates prepared from N2a cells with overexpression of various forms of GFP–PP2A-A (WT, S401A or S401D) and Flag-HDAC4. Lysates were immunoprecipitated with antibodies to Flag or GFP and were blotted with antibodies to phospho-Ser/ThrGln. (**d**) Immunofluorescent images of endogenous or exogenous HDAC4 (green) and PP2A-A (red) in wild-type and *Atm*$^{-/-}$ primary neurons that were in culture for 14 d. White boxes indicate areas magnified in the two single-channel insets. (**e**) Representative images of PP2A distribution in *Atm*$^{+/+}$ and *Atm*$^{-/-}$ cultured neurons with coexpression of GFP–PP2A-A and mCherry–PP2A-C with wild-type, S401A or S401D PP2A-A. Scale bar, 20 μm. (**f**) Effect of the inhibition of ATM activity by caffeine on the localization of GFP-HDAC4 in wild-type embryonic day 16.5 cortical neurons. The five small panels to the right are isolated images of the cell body. The number in each of the small panels represents the time elapsed (h) since the addition of the ATM inhibitor. (**g**) Effect of knocking down PP2A on ATM-deficient GFP-HDAC4 nuclear accumulation in neurons using caffeine plus *Pp2a* shRNA (*shPp2a*). The small panels on the right are as described in **f**. (**h**) Immunofluorescent images of HDAC4 (green) in wild-type and *Atm*$^{-/-}$ primary neurons infected with *shGapdh* or *shPp2a* lentivirus after 3 d in culture and treated with the PP2A-specific inhibitor endothall after 7 d in culture.

S632A mutations) together with *Hdac4* shRNA (which is specific for the mouse *Hdac4* message; **Supplementary Fig. 7a**,**b**) into the cerebella of wild-type and *Atm*$^{-/-}$ mice. One week after injection of the two lentiviruses, we monitored gene transfer by immunohistochemistry using a human-specific antibody to HDAC4 (**Supplementary Fig. 7c**,**d**). In *Atm*$^{-/-}$ cerebellar neurons infected with the two lentiviruses, cytoplasmic HDAC4 (4A) prevented cell cycle reentry and caspase-3 activation (**Fig. 6a**,**b**). By contrast, nuclear HDAC4 (L1062A and 3SA) resulted in cell cycle reentry and caspase-3 activation in both *Atm*$^{-/-}$ and wild-type lentivirus-infected mice (**Fig. 6a**,**b**). Reduced histone acetylation accompanied the higher concentration of nuclear HDAC4 in *Atm*$^{-/-}$ compared to wild-type mice, as confirmed by immunocytochemistry of AcH3 (**Supplementary Fig. 7e**). Overexpression of unmodified human HDAC4 had little effect on either wild-type or *Atm*$^{-/-}$ neurons.

In primary neurons, overexpression of HDAC4 showed that neuronal cell cycle activity was tightly correlated with the presence of HDAC4 nuclear localization (**Supplementary Fig. 6e**,**f**). Cytoplasmic HDAC4 (4A) was protective and prevented *Atm*$^{-/-}$ neuronal cell cycle reentry. This offered us the opportunity to separately test the functions of nuclear and cytoplasmic HDAC4 and relate each to the presence or absence of behavioral abnormalities in *Atm*$^{-/-}$ mice. We co-injected lentiviruses encoding *Hdac4* shRNA (against the *Hdac4* mouse message) together with viruses encoding exclusively cytoplasmic HDAC4 (H4) into mouse cerebellum at postnatal day (P) 18. At this age, the area infected by the virus should be larger than that infected after a comparable injection into an adult mouse. At P39, when the transgene expression is maximal, we subjected the mice to the same training and trials described above. *Atm*$^{-/-}$ mice injected with cytoplasmic 4A were able to stay on the rotarod significantly longer ($P < 0.05$) than *Atm*$^{-/-}$ sham-injected mice (**Fig. 6c**), and the results of the spontaneous locomotor activity observed in the open-field test were consistent with this difference (**Fig. 6d**). The exploratory activity (**Fig. 6e**) of 4A-injected *Atm*$^{-/-}$ mice was significantly greater ($P < 0.05$) than the activity of mice injected with nuclear L1062A (**Fig. 6d**,**e**). L1062A-injected wild-type mice performed substantially worse than sham-injected wild-type mice (**Fig. 6c**–**e**). To test the involvement of PP2A in these results, we injected a lentivirus carrying wild-type PP2A-A or the PP2A-A S401A or S401D isoform into both wild-type and *Atm*$^{-/-}$ cerebella. The PP2A-A S401D mutant blocked HDAC4 nuclear translocation and inhibited cell-cycle and capase-3 activation. However, wild-type PP2A-A and the PP2A-A S401A mutant had little effect on these activities (**Supplementary Fig. 8 a**,**b**). The effect of the PP2A-A S401D mutant on the behavioral abnormalities in the *Atm*$^{-/-}$ mice, however, was modest (**Fig. 6c**–**e**). This might be a result of the broad range of substrates whose phosphorylation would

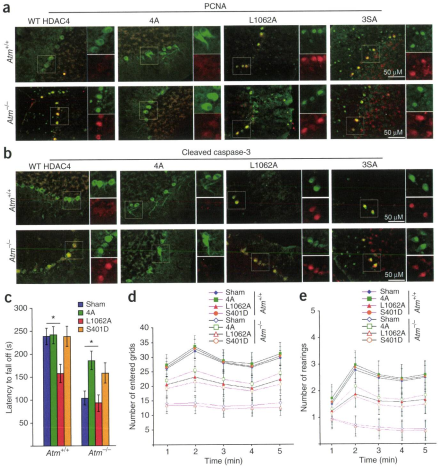

**Figure 6** Blocking nuclear HDAC4 activity rescues the *Atm*$^{-/-}$ phenotype *in vivo* and *in vitro*. (**a**,**b**) Representative images of PCNA-stained and cleaved-caspase-3–stained (red) Purkinje cells showing the effects of the lentiviral delivery of various HDAC4 mutants (green) on degenerative progression in *Atm*$^{-/-}$ mouse cerebella. 4A, NLS (nuclear localization signal) mutant HDAC4 (cytoplasmic); L1062A, nuclear export mutant HDAC4 (nuclear); 3SA, non-phosphorylatable HDAC4 (nuclear). White boxes indicate areas magnified in the two single-channel insets. (**c**) Rotarod tests showing the average latency to fall for wild-type and *Atm*$^{-/-}$ mice after injection of different HDAC4 and S401D lentiviral particles. $^{*}P < 0.001$ by ANOVA. (**d**,**e**) Open-field tests showing the effects of different lentiviral particles of HDAC4 and PP2A-A S401D on the spontaneous locomotor activity (**d**) and exploratory activity (**e**) of *Atm*$^{-/-}$ mice. Each treatment group consisted of 4–6 mice. Data are means ± s.e.m.

be changed by PP2A activity as well as of the indirect nature of the effect of PP2A on the ataxia telangiectasia phenotype.

## DISCUSSION

Our data provide insights into the functions of both ATM and HDAC4 in the health and survival of central nervous system neurons. HDAC4 is normally maintained in the neuronal cytoplasm through its interaction with the 14-3-3 protein. This interaction requires CaMKII- or CaMKIV-mediated HDAC4 phosphorylation. ATM plays an essential part in the cytoplasmic retention of HDAC4, but its action is indirect. It targets neither the CaMK kinases nor HDAC4 itself. Rather, it suppresses the HDAC4 phosphatase, PP2A, by phosphorylating Ser401 of the PP2A-A subunit (**Supplementary Fig. 8c–e**). In ATM deficiency, hypophosphorylated HDAC4 translocates to the nucleus, where it triggers a decrease in histone H3 and H4 acetylation and large-scale changes in the pattern of gene expression.

This study uncovers several major features of the epigenetic landscape of the *Atm*$^{-/-}$ neuronal genome. First, our ChIP analysis showed genome-wide changes in the location of AcH3 and AcH4 in *Atm*$^{-/-}$ mice compared to wild-type mice, which is in contrast to the results seen with unmodified histone H3 or H4. Second, by performing ChIP-seq with antibodies to HDAC4, we provide data in support of the idea that HDAC4 is itself associated with specific genomic locations and that these locations differ depending on the *Atm* genotype of the neuron. Most models of HDAC4 action are based on its binding to and inhibiting proteins, such as MEF2A, thus decreasing their effective concentration at the promoters of key target genes[33,34]. Our ChIP and HDAC4 ChIP-seq data suggest that in the absence of ATM, HDAC4 accumulates in the nucleus, where it directly associates with chromatin. Although HDAC4 may remain associated with MEF2A, CREB or both, which is suggested by the increased binding of nuclear HDAC4 (3SA and L1062A) to MEF2A and CREB in *Atm*$^{-/-}$ mice, it can be described as a full participant in transcriptional suppression. We emphasized this point by comparing the chromatin-binding patterns of HDAC4 with those of AcH3 and AcH4 or of MEF2A and CREB alone. Several neuronal genes, such as *Creb*, *Mef2A* and *Bdnf*, have a reduced association with AcH3 and AcH4 or MEF2A and CREB but show high amounts of association with HDAC4 itself. Third, in the promoter region of each genetic locus we examined, the changes in gene transcription predicted by a more open conformation in the presence of acetylated histone proteins were borne out by the results from both the RT-PCR and western blot analyses. This suggests that key aspects of the neuronal phenotypes observed in ataxia telangiectasia and in *Atm*$^{-/-}$ mice are caused not only by the absence of the ATM kinase activity itself but also by the indirect epigenetic changes in the pattern of histone acetylation and transcription factor inhibition that occur as a result of the presence of nuclear HDAC4.

This concept suggests several new targets for pharmacological intervention in the treatment of ataxia telangiectasia. For example, inhibiting HDAC4 activity and blocking its nuclear accumulation both reverse the altered histone acetylation pattern and rescue various aspects of the neurodegenerative phenotype of mouse ATM-deficient neurons. Particularly noteworthy is the phenotypic rescue of both the neurodegenerative changes of *Atm*$^{-/-}$ cerebellar neurons *in vivo* as well as the improvements in motor behavior when HDAC4 nuclear activity is inhibited or when cytoplasmic HDAC4 is overexpressed. The lentivirus injection studies illustrate this concept on a cell-by-cell basis; the TSA injections make the same point on the level of the whole brain. TSA is admittedly a very broad spectrum HDAC inhibitor, however, the reversal of cell cycle protein expression as a result of TSA treatment was noteworthy. In addition, the failure of sodium butyrate to elicit a comparable effect suggests no involvement of class I HDAC activities. This picture of a two-edged sword—neuroprotection of HDAC4 in the cytoplasm but neurotoxicity of HDAC4 in the nucleus—suggests that it may be more strategic to focus on HDAC4 location rather than HDAC4 activity as the most useful ataxia telangiectasia therapeutic target.

## METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

*Note: Supplementary information is available on the Nature Medicine website.*

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### AUTHOR CONTRIBUTIONS

J.L. and K.H. designed the experiments, analyzed data and wrote the manuscript. C.L.R. and R.P.H. developed, carried out and analyzed data for the ChIP-seq analyses. J.L. and J.C. carried out the immunocytochemistry experiments. J.C. performed all of the qPCR experiments. J.L. and A.K. carried out the mouse cerebellar lentiviral injections. M.S.S., J.L. and A.K. carried out behavioral tests.

### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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# NLRP3 has a protective role in age-related macular degeneration through the induction of IL-18 by drusen components

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**Age-related macular degeneration (AMD) is the leading cause of central vision loss worldwide. Drusen accumulation is the major pathological hallmark common to both dry and wet AMD. Although activation of the immune system has been implicated in disease progression, the pathways involved are unclear. Here we show that drusen isolated from donor AMD eyes activates the NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome, causing secretion of interleukin-1β (IL-1β) and IL-18. Drusen component C1Q also activates the NLRP3 inflammasome. Moreover, the oxidative-stress–related protein-modification carboxyethylpyrrole (CEP), a biomarker of AMD, primes the inflammasome. We found cleaved caspase-1 and NLRP3 in activated macrophages in the retinas of mice immunized with CEP-adducted mouse serum albumin, modeling a dry-AMD–like pathology. We show that laser-induced choroidal neovascularization (CNV), a mouse model of wet AMD, is exacerbated in *Nlrp3*$^{-/-}$ but not *Il1r1*$^{-/-}$ mice, directly implicating IL-18 in the regulation of CNV development. These findings indicate a protective role for NLRP3 and IL-18 in the progression of AMD.**

In the developed world, AMD is the most prevalent cause of legal blindness in older individuals[1,2]. AMD is a progressive disease characterized by the accumulation of focal extracellular deposits on the Bruch's membrane below the retinal pigment epithelium (RPE) in the macula, which is recognized in an eye examination as drusen. The presence of drusen in the macula, the density of the deposits and the area covered by this material are used to define the early stages in the AMD disease process. Individuals with drusen are considered at risk for progressing to the end-stage blinding forms of AMD[3–5]. Geographic atrophy, the end stage of the atrophic 'dry' form of AMD, culminates in vision loss after focal degeneration of the RPE below the fovea[6]. Without the RPE, the foveal cone photoreceptors degenerate, causing central retinal blindness. CNV characterizes the end stage of the exudative 'wet' form of AMD, with new blood vessels breaking through the Bruch's membrane and the RPE that then hemorrhage, causing a blood clot to form between the RPE and the foveal photoreceptors, resulting in immediate blindness[3,4].

AMD is classically multifactorial, involving both environmental and genetic factors[7]. Sequence variants associated with disease susceptibility have now been characterized in a growing number of immune-regulated genes[8–11]. Activation of complement on ocular surfaces is thought to have a major role in the early disease process and results in drusen deposition. However, the mechanisms involved in the initiation of the inflammatory responses observed in the eyes of subjects with AMD are unresolved.

Drusen deposits are particulate protein aggregates that are extracellular in nature. These characteristics typify known activators of the sterile inflammatory response that is mediated by NLRP3. NLRP3 acts as a receptor for 'danger' signals, such as ATP, uric acid crystals, amyloid-like structures and mitochondrial dysfunction[12–15]. These danger signals activate the inflammasome, which is made up of NLRP3, apoptosis-associated speck-like domain containing a caspase-recruitment domain (ASC) and pro–caspase-1, resulting in the cleavage of pro–IL-1β and pro–IL-18 into their mature proinflammatory forms. Furthermore, excessive drusen accumulation can disrupt adjacent RPE cells, which subsequently die by necrosis[16], a cellular process now known to activate the NLRP3 inflammasome[17].

Here we show that drusen isolated from AMD donor eyes can activate the NLRP3 inflammasome. Furthermore, we show that CEP, an oxidative stress–related protein modification commonly found decorating drusen proteins[18,19], can prime the inflammasome. In tandem, we show that the complement component C1Q can activate the NLRP3 inflammasome in a caspase-1–dependent and phagolysosome-dependent manner. We observed activated caspase-1 and NLRP3 in macrophages surrounding the drusen-like lesions in mice immunized with CEP-adducted mouse serum albumin (CEP-MSA), which is a model

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of dry AMD. We also found that NLRP3 affects disease in a mouse model of wet AMD[20,21]. In the absence of NLRP3 in this model, CNV development was exacerbated. We implicate IL-18 as a key regulator of pathological neovascularization and suggest a protective role for the NLRP3 inflammasome in the development of AMD.

## RESULTS

The RPE is a monolayer of cuboidal cells located between the outer retina and the choroid. This melanized neuroepithelium has numerous functions, including (i) the adsorption of scattered and reflected light, (ii) the formation of the outer blood-retinal barrier and (iii) the removal by phagocytosis of the effete tips of the photoreceptor outer segments[22]. Proteomic and immunohistochemical analyses of drusen have identified virtually every protein involved in the complement cascade and the proteins found in amyloid deposits, as well as a number of crystallins, which are proteins synthesized in response to stress[23,24]. Considering the recent discovery that host-derived particulate matter such as cholesterol crystals and amyloid deposits[25,26] can activate the NLRP3 inflammasome, we were interested in determining whether drusen could also initiate the activation of the inflammasome.

### Drusen activates the NLRP3 inflammasome

We first used fundus photography to compare an unaffected eye to the eyes of individuals with either dry or wet AMD (**Fig. 1a**). We found punctate light deposits in the fundus images of the eyes with wet and dry AMD, representing drusen accumulation, and we found subretinal CNV in the image of the eye with wet AMD. We sonicated the isolated drusen to dissociate the sample into small particulate matter (**Fig. 1b**). An SDS-PAGE analysis of the drusen sample showed a cohort of proteins of high molecular weight (greater than 60 kDa) (**Fig. 1c**).

The inflammasome is a multimeric protein complex. Caspase-1 is the cysteine protease that is activated in the inflammasome complex to cleave pro–IL-1$\beta$ and pro–IL-18 into their mature forms. Activation of caspase-1 requires the protein ASC, which forms oligomers to create a platform for the multimeric complex. Normally, ASC is evenly distributed throughout the cell, but once activated, ASC aggregates to a single point known as a 'speck'. We primed bone-marrow–derived macrophages (BMDMs) that stably express yellow fluorescent protein–labeled ASC (YFP-ASC) with lipopolysaccharides (LPSs) and treated them with drusen or transfected them with poly(dA-dT) (positive control). ASC-YFP was difficult to discern in macrophages treated with LPS alone (**Fig. 1d**), however, in LPS-primed macrophages activated with drusen, the formation of intense single fluorescent specks was clearly evident, which is indicative of ASC oligomerization.

The inflammatory response associated with AMD has both a local and a systemic component. We initially tested the ARPE-19 cell line for the presence of NLRP3 and for the ability of the cells to produce IL-1$\beta$ in response to a range of Toll-like receptor (TLR) ligands and activation with ATP. We found that although ARPE-19 cells express NLRP3, the amount of IL-1$\beta$ in these cells was at the lower limit of the assay sensitivity (**Supplementary Fig. 1**). Because of their ability to access the retina in AMD, peripheral myeloid cells are probably the primary source of IL-1$\beta$ and IL-18. We therefore hypothesized that these cells would be key cells of interest in our system. Human peripheral blood mononuclear cells (PBMCs) produced IL-1$\beta$ and IL-18 in response to activation with drusen, even at very low concentrations (**Fig. 1e,f**). We used RPE material that was produced during the dissection of drusen from AMD eyes as a control for these experiments (**Supplementary Fig. 2**). An immunoblot analysis of caspase-1 expression in lysates of cells from

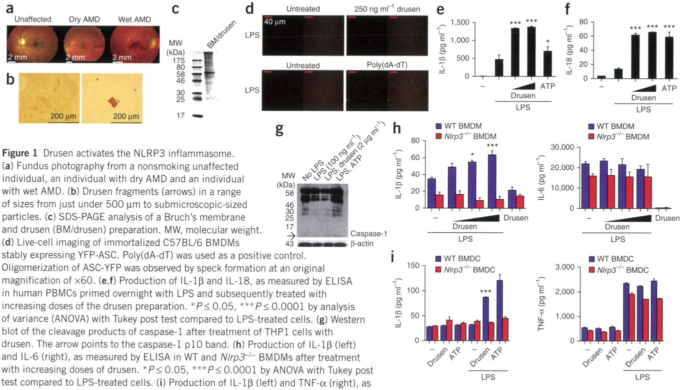


**Figure 1** Drusen activates the NLRP3 inflammasome. (**a**) Fundus photography from a nonsmoking unaffected individual, an individual with dry AMD and an individual with wet AMD. (**b**) Drusen fragments (arrows) in a range of sizes from just under 500 μm to submicroscopic-sized particles. (**c**) SDS-PAGE analysis of a Bruch's membrane and drusen (BM/drusen) preparation. MW, molecular weight. (**d**) Live-cell imaging of immortalized C57BL/6 BMDMs stably expressing YFP-ASC. Poly(dA-dT) was used as a positive control. Oligomerization of ASC-YFP was observed by speck formation at an original magnification of ×60. (**e**,**f**) Production of IL-1$\beta$ and IL-18, as measured by ELISA in human PBMCs primed overnight with LPS and subsequently treated with increasing doses of the drusen preparation. $^{*}P \le 0.05$, $^{***}P \le 0.0001$ by analysis of variance (ANOVA) with Tukey post test compared to LPS-treated cells. (**g**) Western blot of the cleavage products of caspase-1 after treatment of THP1 cells with drusen. The arrow points to the caspase-1 p10 band. (**h**) Production of IL-1$\beta$ (left) and IL-6 (right), as measured by ELISA in WT and *Nlrp3*$^{-/-}$ BMDMs after treatment with increasing doses of drusen. $^{*}P \le 0.05$, $^{***}P \le 0.0001$ by ANOVA with Tukey post test compared to LPS-treated cells. (**i**) Production of IL-1$\beta$ (left) and TNF-$\alpha$ (right), as measured by ELISA in WT and *Nlrp3*$^{-/-}$ BMDCs after treatment with increasing doses of drusen. $^{***}P \le 0.0001$ by ANOVA with Tukey post test compared to LPS-treated cells. All ELISA data are representative of a minimum of three separate experiments carried out in triplicate, and all data are means ± s.e.m.

the human monocytic cell line THP1 after treatment with drusen confirmed increased amounts of the cleaved caspase-1 subunit p10 (**Fig. 1g** and **Supplementary Fig. 3**). Together, these results show that drusen from AMD donor eyes can activate caspase-1 and the ASC inflammasome complex, which in turn results in IL-1β and IL-18 production in PBMCs.

We reasoned that NLRP3 was the probable sensor for drusen-induced inflammasome activation, as it is required for inflammasome activation by particulate matter. We isolated bone marrow from both wild-type (WT) and NLRP3-deficient (*Nlrp3*$^{-/-}$) mice and then used the bone marrow to culture BMDMs and bone-marrow–derived dendritic cells (BMDCs). WT BMDMs and BMDCs produced significantly higher amounts of IL-1β in response to drusen than *Nlrp3*$^{-/-}$ BMDMs and BMDCs (**Fig. 1h,i**), which were unable to promote the production of mature IL-1β in response to drusen. The amounts of IL-6 and tumor necrosis factor α (TNF-α) were unaltered by the presence of drusen, which is indicative of a specific effect of drusen on IL-1β production (**Fig. 1h,i**). These results show that AMD drusen are capable of activating the NLRP3 inflammasome.

## CEP-adducted human serum albumin primes the inflammasome

Up to 65% of the proteins that have been identified in drusen were found in drusen isolated from both AMD and non-diseased donors. However, oxidative protein modifications have also been observed in drusen, including CEP protein adducts. Cumulative oxidative damage contributes to aging and has long been suspected to contribute to the pathogenesis of AMD[27–29]. CEP adducts are uniquely generated from the oxidation of docosahexaenoate-containing lipids and are more abundant in the drusen and serum of subjects with AMD compared to subjects without AMD[19]. Recently, carboxyalkylpyrroles, among them CEP, have been shown to be recognized by TLR2 on endothelial cells[30]. Given that TLR2 activation would prime cells to induce pro–IL-1β, pro–IL-18 and NLRP3, we hypothesized that CEP-adducted proteins in drusen and on the Bruch's membrane could represent a previously unidentified priming agent.

To test this hypothesis, we primed PBMCs with increasing concentrations of CEP-adducted human serum albumin (CEP-HSA) or HSA alone and activated the cells with ATP. The production of IL-1β increased with increasing concentrations of CEP-HSA, but we found no changes in the amount of IL-1β in cells primed with HSA alone (**Fig. 2a**). WT BMDMs primed with CEP-HSA and activated with ATP also produced IL-1β, an effect that we did not observe in *Nlrp3*$^{-/-}$ BMDMs (**Fig. 2b**). To ascertain whether CEP-HSA was priming the cells through TLR2 activation, we primed WT and *Tlr2*$^{-/-}$ BMDMs with HSA or CEP-HSA and activated them with ATP. ATP activation induced increases in IL-1β production in WT but not *Tlr2*$^{-/-}$ BMDMs primed with CEP-HSA. Furthermore, we found no IL-1β induction in BMDMs from either group primed with HSA before ATP activation, again confirming that it is CEP modification that confers the ability to activate TLR2 (**Fig. 2c**). IL-6 concentrations were equivalent in ATP-activated and ATP-unactivated WT cells treated with CEP-HSA, confirming the specificity of the response for IL-1β (**Fig. 2d**).

We measured IL-1β concentrations in LPS-primed WT and *Tlr2*$^{-/-}$ BMDMs activated by ATP to ensure that the *Tlr2*$^{-/-}$ BMDMs were responding optimally (**Supplementary Fig. 4**). To ensure our CEP-HSA was not contaminated with LPS, we isolated BMDMs from C3H/HeN and C3H/HeJ mice. C3H/HeJ mice carry a mutation in their *Tlr4* gene that renders them unresponsive to LPS[31]; the C3H/HeN mice do not have this mutation. C3H/HeJ BMDMs produced IL-1β in response to ATP when primed with CEP-HSA but not when primed with LPS (**Fig. 2e**), indicating that our CEP adduct was LPS free and primed the inflammasome through TLR2 ligation. We detected TNF-α in LPS-primed, but not CEP-primed, C3H/HeN BMDMs (**Fig. 2f**). We further examined the ability of CEP-HSA to prime the NLRP3 inflammasome by measuring ASC-YFP speck formation in CEP-HSA–treated immortalized WT BMDMs. We found focused ASC-YFP specks in BMDMs primed with CEP-HSA and activated with drusen (**Fig. 2g**). Drusen alone seemed to be able to cause the oligomerization of ASC (**Fig. 2g**), implying that alone, drusen could initiate the formation of the multiprotein platform for inflammasome activation. However, we

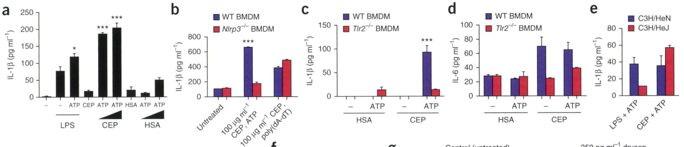
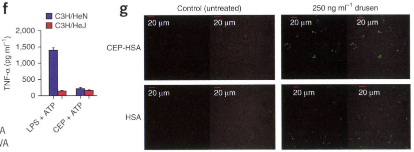


**Figure 2** CEP (CEP-HSA), a component of drusen, can prime the NLRP3 inflammasome. (**a**) Production of IL-1β in human PBMCs primed with LPS, CEP-HSA or HSA alone in increasing doses and subsequently treated with ATP. *$P \leq 0.05$, ***$P \leq 0.0001$ by ANOVA with Tukey post test compared to LPS-treated cells. (**b**) IL-1β production in WT and *Nlrp3*$^{-/-}$ BMDMs primed with CEP-HSA and then activated with either ATP or poly(dA-dT). ***$P \leq 0.0001$ by ANOVA with Tukey post test compared to *Nlrp3*$^{-/-}$ cells. (**c**,**d**) IL-1β and IL-6 production in WT or *Tlr2*$^{-/-}$ BMDMs primed with HSA or CEP-HSA and activated with ATP or left untreated. ***$P \leq 0.0001$ by ANOVA with Tukey post test for WT BMDMs treated with CEP-HSA and ATP compared to *TLR2*$^{-/-}$ BMDMs treated with CEP-HSA and ATP. (**e**,**f**) IL-1β and TNF-α production measured in C3H/HeN BMDMs or C3H/HeJ BMDMs primed with either LPS or CEP and activated with ATP. (**g**) Live-cell imaging of immortalized C57BL/6 BMDMs stably expressing YFP-ASC. Shown are cells primed with CEP-HSA (top) or HSA (bottom), followed by treatment with drusen. Oligomerization of ASC-YFP was observed by speck formation at an original magnification of ×60. All ELISA data are representative of a minimum of three separate experiments carried out in triplicate, and all data are means ± s.e.m.



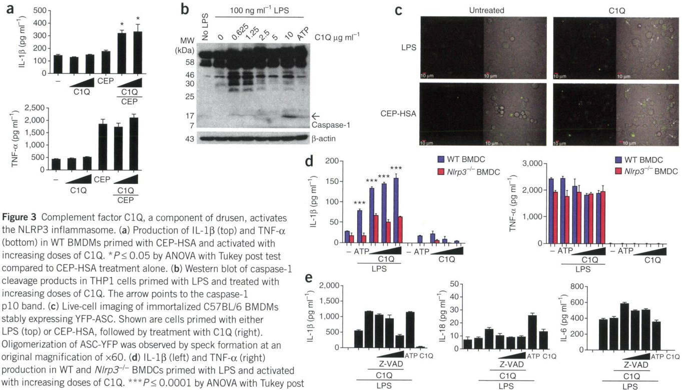


**Figure 3** Complement factor C1Q, a component of drusen, activates the NLRP3 inflammasome. (**a**) Production of IL-1β (top) and TNF-α (bottom) in WT BMDMs primed with CEP-HSA and activated with increasing doses of C1Q. *$P \le 0.05$ by ANOVA with Tukey post test compared to CEP-HSA treatment alone. (**b**) Western blot of caspase-1 cleavage products in THP1 cells primed with LPS and treated with increasing doses of C1Q. The arrow points to the caspase-1 p10 band. (**c**) Live-cell imaging of immortalized C57BL/6 BMDMs stably expressing YFP-ASC. Shown are cells primed with either LPS (top) or CEP-HSA, followed by treatment with C1Q (right). Oligomerization of ASC-YFP was observed by speck formation at an original magnification of ×60. (**d**) IL-1β (left) and TNF-α (right) production in WT and *Nlrp3*$^{-/-}$ BMDCs primed with LPS and activated with increasing doses of C1Q. ***$P \le 0.0001$ by ANOVA with Tukey post test for WT BMDCs compared to *NLRP3*$^{-/-}$ BMDCs for each given treatment. (**e**) IL-1β, IL-18 and IL-6 production in human PBMCs primed with LPS overnight and activated with C1Q, with the addition of increasing doses of Z-VAD before C1Q treatment. All ELISA data are representative of a minimum of three separate experiments carried out in triplicate, and all data are means ± s.e.m.

were unable to consistently detect increases in IL-1β concentration when we treated PBMCs, WT BMDMs or WT BMDCs with drusen alone and assayed them using ELISA.

### Drusen component C1Q activates the inflammasome

Although drusen can distort and eventually damage the retina, as is seen in geographic atrophy[29], not all people presenting with drusen develop vision loss; therefore, it is conceivable that in addition to the particulate nature of drusen causing mechanical insult to the RPE, some component(s) of drusen may be involved in the activation of the inflammasome in a more specific manner. We elected to study C1Q, the primary initiating component of the classical complement pathway, which has been identified in drusen[32]. Because C1Q is an effector of the innate immune system and has the potential to be extremely damaging to host tissue, its presence in drusen is indicative of an earlier or ongoing inflammatory insult. We directly evaluated the ability of C1Q to activate the NLRP3 inflammasome. The addition of C1Q alone to WT BMDMs did not cause the production of IL-1β, however, cells that we primed with CEP-HSA before the addition of C1Q produced significant amounts of IL-1β compared with C1Q alone (**Fig. 3a**). Secretion of the proinflammatory cytokine TNF-α was unchanged after addition of C1Q to CEP-HSA–primed WT BMDMs (**Fig. 3a**), indicating that C1Q was specifically activating the inflammasome and was not involved in the upregulation of proinflammatory cytokines in general. We found cleaved caspase-1 p10 in THP1 human monocytic cells activated with C1Q (**Fig. 3b** and **Supplementary Fig. 5**) and further established that C1Q can cause ASC oligomerization, as we found YFP-ASC specks in concentrated focal points within the cells activated with C1Q after priming with either LPS or CEP-HSA (**Fig. 3c**).

WT BMDCs treated with C1Q produced significantly more IL-1β than *Nlrp3*$^{-/-}$ BMDCs, which did not produce IL-1β in response to C1Q activation (**Fig. 3d**); the amount of TNF-α was the same in both groups of BMDCs after C1Q activation (**Fig. 3d**). To confirm the role of caspase-1, we added a caspase-1 inhibitor, Z-VAD, to human PBMCs before activation with C1Q. Caspase-1 inhibition decreased the production of both IL-1β and IL-18 in a dose-dependent manner (**Fig. 3e**). Together, these results show that C1Q can act as a danger signal that is sensed by the NLRP3 inflammasome. All C1Q isolated from human blood and found in drusen has a propensity to aggregate, which we have shown using a zeta-potential analysis of a solution of C1Q, and we believe this is a key factor in how C1Q can activate the NLRP3 inflammasome (**Supplementary Fig. 6**).

### C1Q inflammasome activation involves the phagolysosome

Deposits of C1Q, along with other complement factors, have been shown to be associated with amyloid structures or their components[33,34]. It is therefore probable that this characteristic of C1Q has a role in the aggregation of drusen. In addition, the function of C1Q in initiating opsonization would assist macrophages as they attempt to phagocytose these particulate drusen deposits. The mechanisms leading to NLRP3 inflammasome activation are unresolved and may depend on the stimulus. One proposed mechanism of NLRP3 activation involves the phagocytosis of particulate structures, leading to lysosomal rupture and the release of lysosomal contents[35]. Another proposed mechanism involves the production of reactive oxygen species (ROS), leading to the activation of the NLRP3 inflammasome through the ROS-sensitive thioredoxin interacting protein (TXNIP)[36].

To determine whether the induction by C1Q of ROS[37,38] was responsible for the inflammasome activation seen in this study, we treated

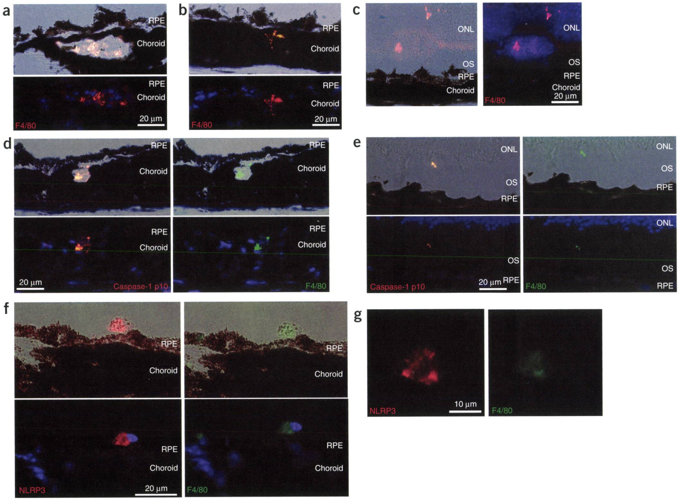


**Figure 4** Cleaved caspase-1 p10 colocalizes with activated macrophages in CEP-MSA–immunized mice. (**a**–**c**) Immunostaining of retinal cryosections of CEP-MSA–immunized mice showing localization of F4/80$^+$ macrophages to regions of the choroid (**a**), extending from the choroid toward the Bruch's membrane (**b**) and present above the RPE in the outer segments (OS) and outer nuclear layer (ONL) of the retina (**c**). Differential contrast images are shown at the top in **a** and **b** and on the left in **c**, and fluorescent images (with F4/80 in red and DAPI in blue) are shown at the bottom in **a** and **b** and on the right in **c**. (**d**,**e**) Colabeling of retinal cryosections of CEP-MSA–immunized mice, with caspase-1 p10 (red) and F4/80 (green) showing colocalization in a macrophage present within and transcending the choroid and the Bruch's membrane (**d**) and a macrophage protrusion in the outer segment of the retina (**e**). (**f**) Colabeling of retinal cryosections of CEP-MSA–immunized mice showing colocalization of NLRP3 (red) and F4/80 (green). (**g**) A higher magnification of the NLRP3 and F4/80 staining in **f**.

PBMCs with the NADPH oxidase inhibitor diphenyliodonium (DPI) before C1Q activation. Inhibition of ROS by DPI had no effect on C1Q-induced IL-1β release (**Supplementary Fig. 7a**). Another alternative mechanism that has been proposed for inflammasome activation is lysosomal instability leading to the leakage of the lysosomal exopeptidase, cathepsin B, into the cytosol, which is then sensed by the components of the inflammasome, leading to its assembly[35]. To determine the role of the phagolysosome in the activation of the inflammasome by C1Q, we used bafilomycin A, an inhibitor that blocks the vacuolar H$^+$ ATPase system that is necessary for lysosomal acidification, and the cathepsin B inhibitor CA-074 Me. Inhibition of either vacuolar H$^+$ ATPase or cathepsin B restricted the C1Q-activated production of IL-1β and IL-18 with no effect on IL-6 production (**Supplementary Fig. 7b**,**c**). This implies that C1Q alters the phagolysosomal process to trigger NLRP3 activation.

## NLRP3 inflammasome is active in CEP-MSA–immunized mice

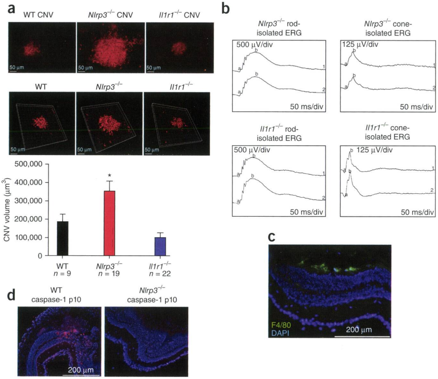
We sought to determine whether the inflammasome is involved in the pathology of a model of dry AMD, the CEP-MSA–immunized mouse model. This mouse develops AMD-like lesions in its retina and RPE after immunization with CEP-MSA.

We analyzed retinal sections of CEP-MSA–immunized mice for the presence of activated macrophages (indicated by F4/80 and CD68 staining), caspase-1 p10 and NLRP3. We found activated macrophages within the choroid and Bruch's membrane in the retinas of these mice (**Fig. 4a**,**b** and **Supplementary Fig. 8**). We also found infiltrating macrophages above the RPE in the outer segments of the retinas (**Fig. 4c**). Staining of these sections showed colocalization of F4/80 with cleaved caspase-1 p10 (**Fig. 4d**,**e**) and NLRP3 (**Fig. 4f**,**g** and **Supplementary Fig. 9**).

## NLRP3 protects against exacerbated laser-induced CNV

A model for wet (exudative) AMD is laser-induced CNV, which is also an ideal model for sterile inflammation[39], probably because of the induction of a necrotic microenvironment within the tissue. Necrotic cells are known to trigger a sterile inflammatory response through the NLRP3 inflammasome[17]. We hypothesized that the NLRP3 inflammasome may have a key role in CNV development in

**Figure 5** NLRP3 is protective against laser-induced CNV lesion formation in an IL-1β–independent manner. (**a**) Laser-induced CNV in WT (top left), *Nlrp3*$^{-/-}$ (top middle) and *Il1r1*$^{-/-}$ (top right) mice showing CNV development after laser burn. Three-dimensional reconstructed images of confocal z-stacks from WT (bottom left), *Nlrp3*$^{-/-}$ (bottom middle) and *Il1r1*$^{-/-}$ (bottom right) mice. Also shown is a CNV volume rendering (bar chart). $*P \leq 0.0496$ by ANOVA followed by Tukey *post-hoc* test. Data are means ± s.e.m. (**b**) ERG analysis of rod and cone function in *Nlrp3*$^{-/-}$ and *Il1r1*$^{-/-}$ mice. The a wave represents hyperpolarization of photoreceptors, and the b wave represents the second-order neuron response. (**c**) Immunostaining showing localization of activated macrophages (F4/80, green) to the site of laser-induced injury in *Nlrp3*$^{-/-}$ mice. (**d**) Immunostaining for cleaved caspase-1 (red) of WT (left) or *Nlrp3*$^{-/-}$ (right) retinal cryosections after injury.



response to localized tissue injury. To test our hypothesis, we administered focal laser burns to the retinas of WT, *Nlrp3*$^{-/-}$ and *Il1r1*$^{-/-}$ mice and assessed their CNV volumes. We found significantly more CNV development and subretinal hemorrhaging in *Nlrp3*$^{-/-}$ mice compared to WT and *Il1r1*$^{-/-}$ mice (**Fig. 5a**). Three-dimensional z-stack confocal volume rendering of the CNV confirmed a significantly greater ($P = 0.0496$) CNV volume in *Nlrp3*$^{-/-}$ mice 6 d after injury compared to WT and *Il1r*$^{-/-}$ mice (**Fig. 5a**). An electroretinographic (ERG) analysis confirmed that both groups of knockout mice had functional rod and cone responses before injury (**Fig. 5b**). We observed activated macrophage infiltration (positive F4/80 immunoreactivity) at the lesion sites in *Nlrp3*$^{-/-}$ mice (**Fig. 5c**), however, cleaved caspase-1 and IL-18 were only evident at the injury sites of WT mice and were notably absent at these sites in *Nlrp3*$^{-/-}$ mice (**Fig. 5d** and **Supplementary Figs. 10** and **11**). These findings describe a role for the NLRP3 inflammasome in the sterile inflammatory response observed in this mouse model of CNV and point toward IL-18 as a regulator of CNV development.

## NLRP3 confers protection against CNV through IL–18

To confirm a role for IL-18 in the NLRP3-mediated protection against exacerbated CNV development, we administered laser-induced CNV to *IL18*$^{-/-}$ mice. We found these mice to have normal retinal function (**Fig. 6a**), as assessed by an ERG analysis. Laser-induced disruption of the Bruch's membrane and a CNV volume quantification in *IL18*$^{-/-}$ mice 6 d after injury showed markedly increased lesions in the CNV from these mice (**Fig. 6b**) than in the CNV from WT mice (**Fig. 6c**). Intravitreally injecting IL-18–neutralizing antibodies subsequent to laser-induced CNV also resulted in significantly ($P = 0.0368$) increased CNV development in injected WT mice compared to WT mice that were not injected (**Supplementary Fig. 12**).

We reasoned that IL-18 might confer its protection through the regulation of vascular endothelial growth factor (VEGF) synthesis. To test this hypothesis, we treated ARPE-19 cells and a mouse brain microvascular endothelial cell line (bEnd.3) with recombinant IL-18 and then analyzed the VEGF concentrations in the growth medium. IL-18 significantly decreased the amount of VEGF secreted by both ARPE-19 and bEnd.3 cells (**Fig. 6d,e**). These findings implicate a

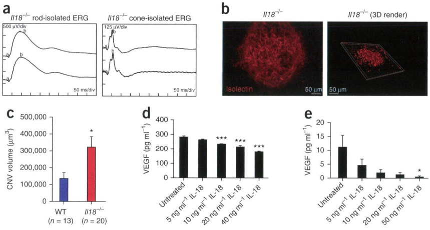


**Figure 6** NLRP3 confers its protection against CNV lesion formation through its role in IL-18 production, which in turn regulates VEGF concentrations. (**a**) ERG analysis of rod and cone function in *Il18*$^{-/-}$ mice. (**b**) Laser induced CNV in *Il18*$^{-/-}$ mice showing CNV development after laser burn (left). Three-dimensional (3D) reconstructed images of confocal z-stacks (right). (**c**) CNV volumes in *Il18*$^{-/-}$ and WT mice (**Fig. 5**). $*P = 0.0292$ by Student's *t* test. (**d**,**e**) The production of VEGF, as assayed by ELISA in ARPE-19 cells (**d**) and bEnd.3 cells (**e**) treated with increasing doses of IL-18 or left untreated. $*P \leq 0.05$, $***P \leq 0.0001$ by ANOVA with Tukey post test compared to untreated cells. ELISA data are representative of a minimum of three separate experiments carried out in triplicate, and all data are means ± s.e.m.

role for IL-18 in the regulation of VEGF expression and could explain the exacerbated CNV in *Nlrp3*$^{-/-}$ and *IL18*$^{-/-}$ mice.

## DISCUSSION

Here we describe a series of observations that could have major implications for the prevention of AMD. Current antibody-based therapies target advanced forms of AMD by inhibiting the bioactivity of VEGF. However, direct and regular intraocular injection of these monoclonal antibodies (Lucentis and Avastin) carries the risk of retinal detachment, hemorrhage and infection[40].

We have shown that drusen isolated from AMD donor eyes can activate the NLRP3 inflammasome. AMD drusen is composed of a collection of protein deposits, many of which are adducted to CEP. Because of its particulate nature, it is possible that drusen from normal donor eyes may also induce inflammasome activation, however the concentrations of normal drusen in the retina, by definition, are lower than the concentrations of AMD drusen, and the biochemical compositions of the two types of drusen are different. These differences are probably crucial for the progression of AMD[41]. A comparison of normal and AMD drusen in relation to inflammasome activation, however, has not yet been made.

We have shown that CEP-HSA can prime the inflammasome through TLR2 activation, providing us with a naturally occurring priming agent that accumulates at focal points at high concentrations within the AMD eye. In the case of NLRP3, the danger signal is usually particulate and is extracellular in nature. C1Q, a component of drusen, has been shown to aggregate in an amyloid-like fashion[42]. We show that C1Q isolated from human blood activates the NLRP3 inflammasome in a manner that is dependent on lysosomal acidification and cathepsin B.

The sterile inflammatory response that occurs in AMD is probably a result of the focal necrosis that occurs in the RPE cells subadjacent to excessive drusen accumulation. Drusen accumulation in the Bruch's membrane is a hallmark feature and diagnostic indicator of early AMD development and is thought to be central to the pathology of the disease. Although we observed inflammasome activation in macrophages associated with AMD-like lesions in CEP-MSA–immunized mice, our observations indicate a protective role for inflammatory processes in the progression to CNV, the exudative form of AMD, and directly oppose the current dogma that is directed at the suppression of inflammatory processes in disease prevention. Indeed, it is now accepted that some amount of inflammation, so-called 'parainflammation', may be beneficial to the host[43,44]. From a clinical perspective, although inflammatory processes have long been associated with AMD pathology and disease development, we suggest that global inhibition of inflammation in the retina in the case of wet AMD would not be a sound therapy. Lending strength to our observations, the results of recent clinical trials of infliximab (Remicade, which targets tumor necrosis factor $\alpha$ (TNF-$\alpha$)) in individuals with wet AMD[45,46] showed that in more than 50% of these subjects, their symptoms were greatly exacerbated. The NLRP3 inflammasome has also recently been shown to confer protection, through IL-18 production, against experimental colitis and colorectal cancer in mice[47,48]. Previous studies indicated that IL-18 has a key role in retinal vascular development. *Il18*$^{-/-}$ mice showed angiectasis and vascular leakage, and VEGF and basic fibroblast growth factor concentrations were also upregulated in the *Il18*$^{-/-}$ mouse retinas[49,50]. Anti-angiogenic roles for IL-18 have also been observed in post-ischemic injury[51] and in the inhibition of tumor angiogenesis[52].

Activation of the NLRP3 inflammasome by drusen suggests that a balance may exist whereby a certain focal amount of drusen is tolerated because of its ability to induce IL-18, which, in turn, may act as an anti-angiogenic effector, maintaining choroidal homeostasis in an inflammatory microenvironment. It is probable that once a critical level of drusen accumulates, its protective role is negated by the excessive damage to the surrounding tissues that it causes. Notably, we have shown that drusen-inducible inflammatory mediators are protective against CNV development and that it is the resultant NLRP3-mediated elevation of IL-18 concentrations that prevents the downstream production of VEGF. Moreover, IL-18 has been shown not to have a role in the development of experimental uveitis, a more conventional model of inflammation, which has direct implications for future forms of therapy deriving from our findings[53]. Overall, our observations directly implicate NLRP3 as a protective agent against the major disease pathology of AMD and suggest that strategies aimed at producing or delivering IL-18 to the eye may be beneficial in preventing the progression of CNV in the context of wet AMD.

## METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

*Note: Supplementary information is available on the Nature Medicine website.*

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### AUTHOR CONTRIBUTIONS

S.L.D. and M.C. conceived of, designed and performed experiments and wrote the paper. E.O. performed experiments. R.G.S. prepared the CEP-HSA. A.M. genotyped all animals reported in the paper and isolated cells. P.F.K. performed ERG analyses. G.J.F., A.-S.K. and M.M.H. contributed to analysis of experiments and data. E.C.L. and L.A.J.O. conceived of experiments and analyzed data. J.G.H. isolated drusen and wrote the paper. P.H. conceived of and designed experiments and wrote the paper.

### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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# Lethal inflammasome activation by a multidrug-resistant pathobiont upon antibiotic disruption of the microbiota

Janelle S Ayres, Norver J Trinidad & Russell E Vance

**The mammalian intestine harbors a complex microbial community that provides numerous benefits to its host. However, the microbiota can also include potentially virulent species, termed pathobiont, which can cause disease when intestinal homeostasis is disrupted. The molecular mechanisms by which pathobionts cause disease remain poorly understood. Here we describe a sepsis-like disease that occurs upon gut injury in antibiotic-treated mice. Sepsis was associated with the systemic spread of a specific multidrug-resistant *Escherichia coli* pathobiont that expanded markedly in the microbiota of antibiotic-treated mice. Rapid sepsis-like death required a component of the innate immune system, the Naip5-Nlrc4 inflammasome. In accordance with Koch's postulates, we found the *E. coli* pathobiont was sufficient to activate Naip5-Nlrc4 and cause disease when injected intravenously into unmanipulated mice. These findings reveal how sepsis-like disease can result from recognition of pathobionts by the innate immune system.**

The intestinal microbiota is composed of diverse microbial species that provide numerous benefits to their hosts[1]. However, the microbiota can also contain pathobionts that can cause disease if not properly constrained[1,2]. A major mechanism constraining pathobionts is believed to be competition or suppression by the healthy microbiota[1]. Indeed, disruption of the healthy microbiota (for example, by antibiotic treatment) can elicit pathobiont virulence and disease[1,3–6]. Although pathobionts are believed to contribute to numerous disease states[7–11], it has been challenging to identify the causal molecular mechanisms that are responsible for disease *in vivo*[12–15].

A key orchestrator of innate immune responses are inflammasomes, multiprotein complexes that detect infection in the cytosol and are required for activation of the caspase-1 protease, and downstream secretion of two of its substrates, the proinflammatory cytokines interleukin-1β (IL-1β) and IL-18 (ref. 16). Several distinct inflammasomes have been described, including the Naip5-Nlrc4 inflammasome, which detects flagellin proteins from diverse bacteria[17,18]. Recently, several reports have established a protective role for inflammasomes in regulating microbiota composition and tissue reparative and regenerative responses to intestinal injury[13,19–22]. Although inflammasome responses in these models are protective against disease, it is also well recognized that excessive inflammation has the potential to be pathological. However, a potential pathological role of inflammasome activation in response to a disrupted intestinal flora has not yet been investigated.

Here we find that disruption of the microbiota by antibiotics predisposes mice to a lethal sepsis-like disease upon intestinal injury. Death is associated with the systemic infection with a multidrug-resistant *E. coli* pathobiont and requires activation of the Naip5-Nlrc4 inflammasome. In accordance with Koch's postulates, intravenous injection of the *E. coli* pathobiont into normal mice recapitulates the rapidly fatal Naip5-Nlrc4–dependent sepsis. Our results reveal a molecular mechanism by which disruptions of intestinal homeostasis can result in aberrant pathobiont-induced innate immune signaling and rapid sepsis-like death.

## RESULTS

### Antibiotics and intestinal injury results in sepsis

To study the innate immune response to a disrupted intestinal microbiota, we established a disease model that couples antibiotic treatment with dextran sulfate sodium (DSS)-induced intestinal injury. DSS is toxic to colonic epithelial cells and typically causes a colitis-like disease[23] characterized by severe weight loss, colonic bleeding and colonic shortening (**Supplementary Fig. 1a**,**b**). We wanted to understand how antibiotic-induced disruption of the microbiota (dysbiosis) would influence DSS-induced disease. Oral administration of a broad-spectrum antibiotic cocktail composed of ampicillin, vancomycin, neomycin and metronidazole (AVNM) to colony-born C57BL/6 wild-type mice resulted in a change in the microbiota composition and a reduction in the amount of 16S rDNA gene copy number along the intestinal tract (**Supplementary Fig. 1c–e**). After this initial treatment, we gave the mice AVNM plus 5% DSS and monitored survival. Consistent with previous reports[24], mice treated with AVNM showed faster death kinetics in response to DSS compared to wild-type littermates that received DSS only (**Fig. 1a** and **Supplementary Fig. 1f**). Notably, ampicillin alone was sufficient to increase mortality in response to DSS treatment (**Supplementary Fig. 1g**,**h**). In contrast, mice treated with the broad-spectrum antibiotic streptomycin and DSS showed similar death kinetics to DSS-treated littermates that were not treated with antibiotics (**Fig. 1b**).

Although DSS typically induces colitis, we found that AVNM- plus DSS-treated colony-born mice did not show the hallmark symptoms

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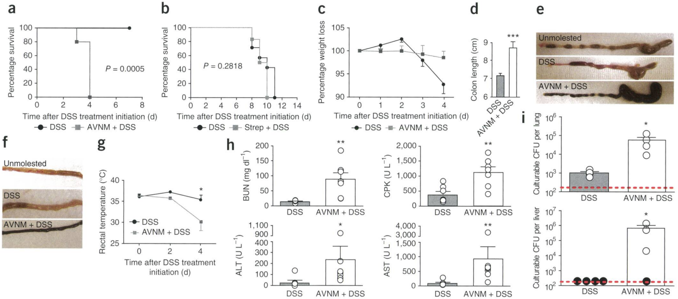


**Figure 1** Antibiotic treatment plus intestinal injury triggers a sepsis-like syndrome in wild-type mice. (**a**) Survival of wild-type male and female mice that received AVNM plus 5% DSS ($n = 5$) or 5% DSS only ($n = 8$). $P = 0.0005$ by log-rank analysis. (**b**) Survival of wild-type male and female mice that received streptomycin (Strep) plus 5% DSS ($n = 6$) or 5% DSS only ($n = 7$). $P = 0.2818$ by log-rank analysis. (**c**) Weight loss of wild-type male and female mice treated with 5% DSS compared to littermates that received drinking water supplemented with AVNM plus 5% DSS. Error bars indicate s.d.; DSS only ($n = 9$); AVNM plus DSS ($n = 9$). (**d**) Length of colons from wild-type male and female mice treated with 5% DSS compared to littermates that received drinking water supplemented with AVNM plus 5% DSS. Error bars indicate s.d. ***$P = 0.0003$ by Student's $t$ test. DSS only ($n = 4$); AVNM plus DSS ($n = 4$). (**e**) Representative cecums and colons at 4 d after DSS treatment initiation of wild-type male mice treated with 5% DSS or AVNM plus 5% DSS or water-treated (unmolested) control littermates. (**f**) Representative images of small intestine at 4 d after DSS treatment initiation of wild-type male mice treated with 5% DSS or AVNM plus 5% DSS or water-treated (unmolested) control littermates. (**g**) Rectal temperature of wild-type male and female mice treated with AVNM plus DSS compared to littermates that received 5% DSS only. Error bars represent s.d. *$P < 0.05$ by Student's $t$ test. DSS only ($n = 8$); AVNM plus DSS ($n = 5$). (**h**) Serum concentrations of blood urea nitrogen (BUN), creatine phosphokinase (CPK), alanine transaminase (ALT) and aspartate transaminase (AST) at 3 d after DSS treatment initiation of wild-type male and female mice. Data are representative of two combined experiments. **$P < 0.01$ and *$P < 0.05$ by Mann-Whitney test. DSS only ($n = 7$); AVNM plus DSS ($n = 7$). Error bars indicate s.d. (**i**) Amounts of culturable bacteria in the lung and liver of wild-type male and female mice 3 d after DSS treatment initiation. Data are representative of three independent experiments. *$P < 0.05$ by Student's $t$ test and $n = 4$ for both conditions. Error bars indicate s.d. Red horizontal dashed lines indicates limit of detection, and black filled circles indicate individual mice below the limit of detection. CFU, colony-forming units.

of colitis (for example, weight loss, colonic inflammation and shortening and poor stool pellet formation, as seen in mice treated with DSS alone; **Fig. 1c–e** and data not shown). Rather, AVNM- plus DSS-treated mice showed bleeding in the small intestine and hypothermia (**Fig. 1f,g** and **Supplementary Fig. 2a**), as well as multiple organ damage and elevated serum concentrations of proinflammatory cytokines including tumor necrosis factor-α (TNF-α) and IL-6 (**Fig. 1h** and data not shown). These results suggest that AVNM-induced changes to the microbiota trigger a systemic disease distinct from colitis in response to intestinal injury. Hypothermia and multiple organ damage are associated with sepsis in mice[25,26]. In addition, we found that antibiotic-treated mice showed significantly higher colonization of culturable bacteria in the lung ($P < 0.05$) and the liver ($P < 0.05$) compared to non–antibiotic-treated mice (**Fig. 1i** and **Supplementary Fig. 2b,c**).

To determine whether the sepsis-like disease was specific to mice from our facility, we analyzed disease progression in AVNM- plus DSS-treated C57BL/6 specific pathogen–free (SPF) mice from Jackson Laboratory (Jax mice) and Taconic Farms (Taconic mice). AVNM- plus DSS-treated Taconic mice developed symptoms associated with a sepsis-like disease, characterized by hypothermia, and increased mortality compared to Taconic mice treated with only DSS. Furthermore, AVNM attenuated DSS-induced colitis symptoms in Taconic mice (**Supplementary Fig. 3a,b** and data not shown). In contrast, disease in AVNM- plus DSS-treated Jax mice appeared to develop similarly to that in Jax mice treated with only DSS (**Supplementary Fig. 3c–e** and data not shown). Thus, it seems that SPF mice from different facilities show distinct disease symptoms in response to treatment with AVNM plus DSS.

## Antibiotic-induced expansion of drug-resistant *E. coli*

Several nonexclusive models could explain how alterations in the microbiota can trigger disease. For example, antibiotic treatment could eliminate members of the microbiota that elicit protective host responses[24]. In addition, antibiotics could result in the overgrowth of AVNM-resistant pathobionts. Consistent with the latter possibility, after 7 d of AVNM treatment, colony-born wild-type mice harbored an expanded population of AVNM-resistant bacteria along the intestinal tract (**Fig. 2a**). We observed a similar expansion of an AVNM-resistant population in Taconic mice but no detectable levels of culturable AVNM-resistant bacteria in Jax mice (**Supplementary Fig. 3**). Furthermore, we found that the AVNM-resistant bacteria colonized extraintestinal tissues of colony-born wild-type mice treated with AVNM plus DSS (**Fig. 2b,c** and **Supplementary Fig. 2**). Thus, sepsis-like disease correlated with the intestinal expansion and extraintestinal colonization of AVNM-resistant bacteria.

We obtained an AVNM-resistant isolate from our mice that we identified as an *E. coli* O21:H+ by16S rDNA sequencing, biochemical characterization and serotyping (**Fig. 2d,e** and data not shown). An expansion of intestinal Enterobacteriaceae species in response to

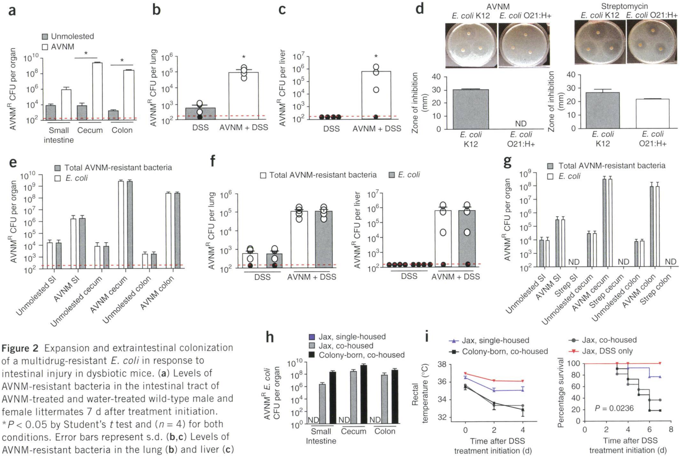


**Figure 2** Expansion and extraintestinal colonization of a multidrug-resistant *E. coli* in response to intestinal injury in dysbiotic mice. (**a**) Levels of AVNM-resistant bacteria in the intestinal tract of AVNM-treated and water-treated wild-type male and female littermates 7 d after treatment initiation. $*P < 0.05$ by Student's *t* test and ($n = 4$) for both conditions. Error bars represent s.d. (**b**,**c**) Levels of AVNM-resistant bacteria in the lung (**b**) and liver (**c**) of AVNM-treated and water-treated wild-type male and female littermates 3 d after DSS treatment initiation. $*P < 0.05$ by Student's *t* test and ($n = 4$) for both conditions. Error bars represent s.d. (**d**) Representative images of disc diffusion assays and quantification of the zone of inhibition to determine the susceptibility of an *E. coli* O21:H+ isolate to AVNM or streptomycin. *E. coli* K12 was used as a control. Error bars indicate s.d. ND, not detected. (**e**) Intestinal levels of total AVNM-resistant bacteria and AVNM-resistant *E. coli* O21:H+ in AVNM-treated wild-type male and female mice ($n = 4$) compared to littermates that received a water control ($n = 4$). SI, small intestine. Error bars represent s.d. (**f**) Lung and liver amounts of total AVNM-resistant bacteria and AVNM-resistant *E. coli* O21:H+ in AVNM-plus DSS-treated wild-type male and female mice ($n = 4$) compared to littermates that received DSS only ($n = 4$). Error bars represent s.d. (**g**) Intestinal amounts of AVNM-resistant *E. coli* and streptomycin-resistant *E. coli* in AVNM- or streptomycin-treated wild-type male and female mice compared to wild-type littermates that received a water control. Unmolested mice ($n = 4$); AVNM-treated mice ($n = 4$); streptomycin-treated mice ($n = 4$). Error bars represent s.d. (**h**) Intestinal amounts of AVNM-resistant *E. coli* in single-housed female Jax mice and Jax mice co-housed with female colony-born (CB) mice after 7 d of AVNM treatment. Jax single-housed ($n = 3$); Jax co-housed ($n = 3$); CB co-housed ($n = 3$). Error bars represent s.d. (**i**) Temperature and survival of AVNM- plus DSS-treated female single-housed Jax mice and Jax mice co-housed with female colony-born mice. For survival, $P = 0.0236$ by log-rank analysis. Data represent two combined experiments; Jax single-housed ($n = 11$); Jax co-housed ($n = 11$); CB co-housed ($n = 11$); DSS only ($n = 5$). For **a**–**c**, **e** and **f**, red horizontal dashed line represents the limit of detection, and black filled circles represent individual mice below the limit of detection. ND, not detected.

antibiotic treatment is consistent with previous reports[27]. AVNM-resistant *E. coli* was also recovered from the lung, liver, spleen and kidney of AVNM- plus DSS-treated colony-born mice (**Fig. 2f** and **Supplementary Fig. 2e,f**). To determine whether *E. coli* was the predominant systemic AVNM-resistant species in our mice, we performed 16S rDNA cloning and sequencing analyses of livers and spleens from AVNM- plus DSS-treated colony-born mice. Although this approach identified clones representing multiple taxa, *Escherichia* was the most abundant taxon identified, consistent with our culturing experiments. In addition, *E. coli* was the only taxon recovered from all tissues from AVNM- plus DSS-treated mice analyzed (data not shown). We also identified AVNM-resistant *E. coli* in the intestines of Taconic mice (**Supplementary Fig. 3**).

*E. coli* O21:H+ was present in the microbiota of unmanipulated mice (**Fig. 2e**) but was not abundant, suggesting it was not able to compete efficiently for intestinal colonization with other members of the flora in the absence of antibiotics. Consistent with this hypothesis, we found that 1 week after cessation of AVNM treatment the aberrant expansion of *E. coli* O21:H+ was suppressed (**Supplementary Fig. 4a**), and such mice no longer developed sepsis in response to DSS (**Supplementary Fig. 4b,c**).

As streptomycin-treated mice did not show rapid sepsis-like death in response to DSS (**Fig. 1b**), we hypothesized that our AVNM-resistant *E. coli* isolate is sensitive to streptomycin. Indeed, we found that our *E. coli* O21:H+ isolate is sensitive to streptomycin and is eliminated from mice by streptomycin treatment (**Fig. 2d,g**). Taken together, our data clearly demonstrate that the sensitivity of mice to AVNM plus DSS and development of sepsis is associated with intestinal overgrowth and extraintestinal colonization of AVNM-resistant *E. coli* O21:H+.

Because Jax mice do not seem to be colonized with AVNM-resistant *E. coli* O21:H+, we could use them to determine whether this bacterium and its associated disease phenotype are transmissible. We housed Jax mice with colony-born mice and treated them with AVNM for 7 d, after which the Jax mice showed AVNM-resistant *E. coli* colonization levels comparable to those in the colony-born mice. In contrast, single-housed Jax mice had no detectable AVNM-resistant *E. coli* (**Fig. 2h**). Upon induction of intestinal injury by DSS, AVNM-treated Jax mice housed with colony-born mice showed symptoms of sepsis-like disease and mortality, similar to colony-born mice, and unlike single-housed Jax mice (**Fig. 2i**). These results indicate that *E. coli* O21:H+ and its associated sepsis phenotype are transmissible.

### Systemic *E. coli* infection is sufficient to induce sepsis

To determine whether our *E. coli* O21:H+ isolate is sufficient to cause sepsis-like disease in wild-type mice, we orally infected AVNM-treated Jax mice with *E. coli* O21:H+. Oral administration of *E. coli* O21:H+ to AVNM-treated Jax mice resulted in high levels of intestinal colonization (**Supplementary Fig. 4d**). Furthermore, AVNM- plus DSS-treated, *E. coli* O21:H+–colonized Jax mice developed hypothermia that was associated with reduced colitis symptoms compared to AVNM- plus DSS-treated Jax mice that received an oral PBS challenge (**Supplementary Fig. 4e**).

To determine whether the *E. coli* O21:H+ isolate is sufficient to cause disease in wild-type mice with a normal (non–antibiotic-treated) microbiota, we intravenously injected normal colony-born and Jax C57BL/6 mice with live or dead *E. coli* O21:H+ and monitored disease. Mice were highly susceptible to a systemic challenge of live but not heat-killed bacteria, as indicated by increased mortality (**Fig. 3a**). In addition, systemic live *E. coli* O21:H+ infection was sufficient to recapitulate the specific pathology associated with AVNM plus DSS treatment (**Fig. 3b,c**). Furthermore, live *E. coli*–infected mice showed tissue colonization patterns and levels similar to those observed in AVNM- plus DSS-treated wild-type mice (**Figs. 1i**, and **3d** and **Supplementary Fig. 2b,c**). Thus, in fulfillment of Koch's postulates, a live systemic infection with *E. coli* O21:H+ is sufficient to recapitulate the mortality and morbidity associated with AVNM- plus DSS-induced sepsis.

### *E. coli* O21:H+ harbors virulence factors

*E. coli* O21 strains have been isolated from patients with extraintestinal infections including bacteremia and sepsis[28–32]. We therefore hypothesized that the *E. coli* pathobiont encodes virulence factors mediating its pathogenicity in wild-type mice. Consistent with this hypothesis, disease was attenuated in mice intravenously infected with a nonpathogenic *E. coli* K12 strain (MG1655) as compared to our *E. coli* O21:H+ isolate (**Fig. 3e,f**), suggesting that detection of viable, nonpathogenic strains of *E. coli* by the immune system[33] is not sufficient to trigger sepsis-like disease in wild-type mice.

To identify virulence-associated determinants, we sequenced the genome of our *E. coli* O21:H+ isolate and aligned the reads to reference *E. coli* genomes (**Supplementary Table 1**). The *fliC* gene, encoding flagellin (H antigen), was most similar to that of H21 isolates (**Supplementary Fig. 5a**). In addition, alignment to the *E. coli* O157:H7 strain Sakai reference genome revealed that our O21:H+ isolate harbors a cluster of genes with similarity to the ETT2 type III secretion system[34] (**Supplementary Fig. 5b**). Many septicemic

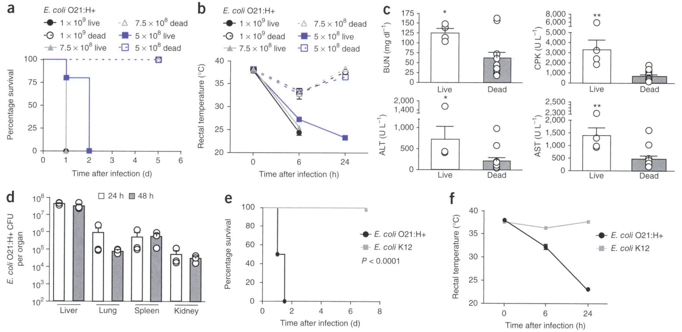
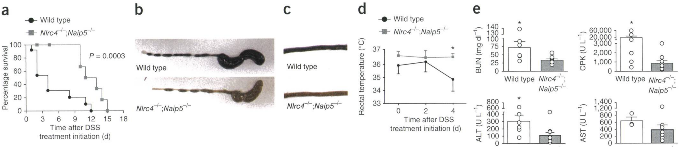


**Figure 3** Systemic *E. coli* O21:H+ infection is pathogenic in wild-type mice. (**a**,**b**) Survival (**a**) and rectal temperature (**b**) of wild-type female mice injected with $1 \times 10^9$, $7.5 \times 10^8$ or $5 \times 10^8$ live or dead *E. coli* O21:H+. For **a**, $P < 0.0001$ for live versus dead comparisons by log-rank analysis, $n = 5$ females for all conditions. For **b**, at 6 h after infection, $1 \times 10^9$ live versus $1 \times 10^9$ dead $P = 0.0079$, $7.5 \times 10^8$ live versus $7.5 \times 10^8$ dead $P = 0.0079$; at 24 h after infection, $5 \times 10^8$ live versus $5 \times 10^8$ dead $P = 0.0195$. $n = 5$ for all conditions. Error bars represent s.d. (**c**) Serum concentrations of BUN, CPK, ALT and AST in wild-type female mice = inoculated with $5 \times 10^8$ live or dead *E. coli* O21:H+ at 24 h after infection. $n = 4$ for live infection and $n = 12$ females for dead infection. $^*P < 0.05$ and $^{**}P < 0.01$ by Student's *t* test, and error bars indicate s.d. (**d**) *E. coli* O21:H+ CFUs in liver, lung, spleen and kidneys of wild-type female mice infected with $5 \times 10^8$ live bacteria at 24 and 48 h after infection. $n = 3$ at both 24 h and 48 h after infection, and error bars indicate s.d. (**e**,**f**) Survival (**e**) and temperature (**f**) of wild-type female mice infected with $5 \times 10^8$ live *E. coli* O21:H+ or *E. coli* K12. For **e**, $P < 0.0001$ by log-rank analysis. $n = 8$ for both *E. coli* O21:H+ and *E. coli* K12 injections. For **f**, $n = 8$ for both *E. coli* O21:H+ and *E. coli* K12 injections. Error bars represent s.d.



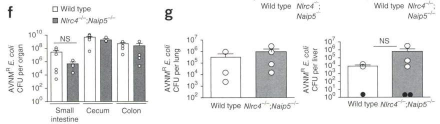


**Figure 4** Naip5-Nlrc4 mediates the pathogenesis of a sepsis-like syndrome in response to intestinal injury in dysbiotic mice. (**a**–**e**) Wild-type and *Nlrc4*$^{-/-}$;*Naip5*$^{-/-}$ male and female fostermates were treated with AVNM plus 5% DSS, and disease severity was monitored. (**a**) Survival Kaplan-Meier plot is representative of two experiments, and $P = 0.0003$ by log-rank analysis. Wild type ($n = 13$); *Nlrc4*$^{-/-}$;*Naip5*$^{-/-}$ ($n = 14$). (**b**) Representative images of cecums and colons from wild-type and mutant female fostermates at 3 d after DSS treatment initiation. (**c**) Representative images of small intestines from wild-type and mutant female fostermates at 3 d after DSS treatment initiation. (**d**) Rectal temperatures. Data are representative of three experiments. $P < 0.05$ by Student's *t* test. Wild type ($n = 10$); *Nlrc4*$^{-/-}$;*Naip5*$^{-/-}$ ($n = 7$). (**e**) Serum BUN, CPK, AST and ALT concentrations at 2 d after DSS treatment initiation. Data are from three experiments and analyzed by Mann-Whitney *t* test. $^*P < 0.05$. Wild type ($n = 3$); *Nlrc4*$^{-/-}$;*Naip5*$^{-/-}$ ($n = 3$). Error bars indicate s.d. (**f**) Amounts of AVNM-resistant *E. coli* in the intestinal tracts of wild-type and *Nlrc4*$^{-/-}$*Naip5*$^{-/-}$ male and female fostermates after 7 d of AVNM treatment. Wild type ($n = 6$); *Nlrc4*$^{-/-}$;*Naip5*$^{-/-}$ ($n = 4$). NS, not significant. Error bars indicate s.d. (**g**) Lung and liver amounts of total AVNM-resistant *E. coli* O21:H+ in AVNM + DSS-treated wild-type mice and AVNM- plus DSS-treated *Nlrc4*$^{-/-}$;*Naip5*$^{-/-}$ male and female fostermates 3 d after DSS treatment initiation. Black filled circles represent tissues isolated from mice that had no bacteria above the limit detection. Error bars represent s.d. NS, not significant.

*E. coli* isolates encode a unique version of the ETT2 gene cluster called ETT2$_{\text{sepsis}}$[35] that contains a large deletion in the *eiv* gene cluster (cluster E in **Supplementary Fig. 5b**), creating deletions and truncations for the majority of *eiv* genes[35]. Despite this, ETT2$_{\text{sepsis}}$ is essential for sepsis pathogenesis, as deletion of genes within the ETT2$_{\text{sepsis}}$ gene cluster render septicemic *E. coli* avirulent[35]. Notably, we were unable to align the genomic content of the *E. coli* O21:H+ genome to the 8.7-kb region of the ETT2 gene cluster that contains the *eiv* genes, suggesting that *E. coli* O21:H+ harbors a form of the ETT2$_{\text{sepsis}}$ gene cluster (**Supplementary Fig. 5b**).

We also found that *E. coli* O21:H+ encodes additional factors associated with extraintestinal *E. coli* clinical isolates including those important for adhesion (*papC*, the *fim* gene cluster and the *pil* gene cluster), internalization (the *csg* genes) and iron acquisition (enterobactin and ferrichrome gene clusters)[35–43]. The genome also encodes *malX* (marker for pathogenicity-associated island from strain CFT073)[38].

## Naip5-Nlrc4 inflammasome mediates AVNM/DSS-induced sepsis

As pathogenic *E. coli* were previously reported to activate inflammasomes containing Naip and Nlrc4 proteins[44,45], we hypothesized that the Naip5-Nlrc4 inflammasome might mediate disease in our sepsis model. Indeed, we found AVNM- plus DSS-induced disease progression was highly attenuated in *Nlrc4*$^{-/-}$;*Naip5*$^{-/-}$ mice (**Fig. 4a**–**e**). Notably, AVNM-treated *Nlrc4*$^{-/-}$ *Naip5*$^{-/-}$ mutant mice showed similar levels of AVNM-resistant *E. coli* overgrowth in the intestinal tract as wild-type fostermates (wild-type pups that nursed on the mother of mutant pups) before DSS treatment, implying that inflammasome deficiency did not exert its protective effects by modulating bacterial growth (**Fig. 4f** and **Supplementary Fig. 6a**,**b**). Moreover, the organs of AVNM-plus DSS-treated *Nlrc4*$^{-/-}$;*Naip5*$^{-/-}$ mice were colonized with similar levels of AVNM-resistant *E. coli* as wild-type mice (**Fig. 4g** and **Supplementary Fig. 6c**,**d**). These data indicate that the absence of Naip5-Nlrc4 function protects AVNM- plus DSS-treated mice from developing sepsis at a step after extraintestinal dissemination of *E. coli* O21:H+. As *Nlrc4*$^{-/-}$;*Naip5*$^{-/-}$ mice eventually succumb to AVNM- plus DSS-induced sepsis, it is likely that additional host factors also contribute to disease.

The Naip5-Nlrc4 inflammasome generally detects flagellin translocated to the host cell cytosol via type III or IV secretion systems[44]. Because *E. coli* O21:H+ encodes a type III secretion system and a functional flagellin (FliC) (**Fig. 5a** and **Supplementary Fig. 5**) similar to that of other pathogenic *E. coli* species, we hypothesized that the cytosolic presence of flagellin from this pathobiont can activate the Naip5-Nlrc4 inflammasome. We tested this hypothesis using a previously described retroviral 'lethality' assay[44] in which flagellin is expressed from a retroviral promoter directly in host cells, allowing for analysis of the effects of flagellin in the absence of other bacterial factors. We retrovirally transduced wild-type, *Nlrc4*$^{-/-}$ and *Naip5*$^{-/-}$ bone marrow–derived macrophages with *fliC*-IRES-GFP, control GFP or control *Legionella pneumophila* flagellin (*flaA*-IRES-GFP). Activation of Naip5-Nlrc4 by flagellin results in cell death, as indicated by the loss of GFP-positive cells. As previously reported[46], cells expressing *L. pneumophila flaA*-IRES-GFP could only be recovered from *Nlrc4*$^{-/-}$ macrophages (**Fig. 5b**,**c**). Detection of FlaA by Naip6 probably accounts for the residual responsiveness of *Naip5*$^{-/-}$ cells. However, we were able to recover cells expressing *E. coli* O21:H+ *fliC*-IRES-GFP from both *Nlrc4*$^{-/-}$ and *Naip5*$^{-/-}$ macrophages but not wild-type macrophages (**Fig. 5b**,**c**). These data suggest that cytosolic flagellin derived from *E. coli* O21:H+ activates the Naip5-Nlrc4 inflammasome and triggers host cell death.

## Naip5-Nlrc4 inflammasome reduces host tolerance of *E. coli*

To address Koch's postulates, we infected wild-type and *Nlrc4*$^{-/-}$;*Naip5*$^{-/-}$ mice intravenously with *E. coli* O21:H+. Systemic *E. coli* O21:H+ infection was significantly less lethal in the absence of

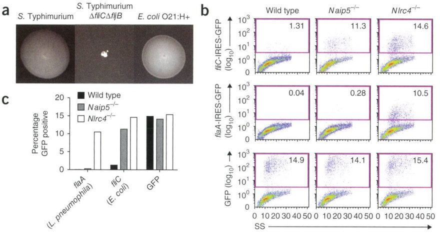


**Figure 5** *E. coli* O21:H+ activates the Naip5-Nlrc4 inflammasome. (**a**) Motility agar was inoculated with wild-type, motile *Salmonella enterica* var. Typhimurium (*S.* Typhimurium) LT2 as a positive control, flagellin-deficient *S.* Typhimurium *ΔfliCΔfljB* as a negative control or *E. coli* O21:H+ and incubated at 37 °C overnight. Representative images of the diffusion of motile bacteria are shown. (**b**) Retroviral constructs expressing *fliC* from *E. coli* O21:H+ or *flaA* from *L. pneumophila* followed by an IRES-GFP element or GFP alone were transduced into wild-type, *Naip5*$^{-/-}$ or *Nlrc4*$^{-/-}$ macrophages, and the percentage of GFP-positive cells (numbers indicated in upper right corners of individual graphs) was enumerated by flow cytometry 4 d after transduction. SS, side scatter. (**c**) The percentage of cells expressing GFP from the retroviral lethality assay in **b** is indicated and charted. Data are representative of three independent experiments.

Naip5-Nlrc4 function ($P = 0.0001$) (**Fig. 6a** and **Supplementary Fig. 7a,b**). The absence of Naip5-Nlrc4 function also alleviated the pathologies associated with *E. coli* O21:H+ infection, including small intestinal bleeding, multiple organ damage and hypothermia (**Fig. 6b–d**). Furthermore, *E. coli* O21:H+ infection was attenuated in *Casp1*$^{-/-}$ (caspase 1 deficient) and *Il1b*$^{-/-}$ mice (**Fig. 6e** and **Supplementary Fig. 7c,d**). Additionally, systemic administration of IL-1 receptor–specific antibody protected mice from AVNM- plus DSS-induced disease (**Fig. 6f**). Thus, the absence of IL-1β is sufficient to protect mice from a systemic inflammatory response induced by a systemic *E. coli* O21:H+ infection (**Fig. 6e** and **Supplementary Fig. 7d**). Taken together, our data clearly indicate that Naip5-Nlrc4 inflammasome signaling through IL-1β in response to a systemic *E. coli* O21:H+ infection leads to sepsis.

The greater susceptibility of wild-type (as compared to *Nlrc4*$^{-/-}$;*Naip5*$^{-/-}$) mice to *E. coli* O21:H+ infection was not associated with higher levels of extraintestinal bacterial colonization (**Fig. 6g**). In fact, colonization levels were similar in both genotypes.

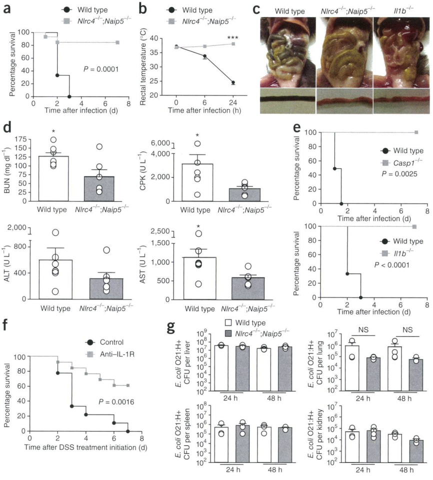


**Figure 6** The Naip5-Nlrc4 inflammasome reduces host tolerance of a systemic *E. coli* infection. (**a–d**) Female wild-type and *Naip5*$^{-/-}$;*Nlrc4*$^{-/-}$ mutant mice were infected with $5 \times 10^8$ live *E. coli*, and disease was monitored. (**a**) Survival; $P = 0.0001$ by log-rank analysis. Wild-type ($n = 9$); *Nlrc4*$^{-/-}$;*Naip5*$^{-/-}$ ($n = 11$). (**b**) Rectal temperature; wild-type ($n = 9$); *Nlrc4*$^{-/-}$;*Naip5*$^{-/-}$ ($n = 11$). ***$P = 0.0003$ by Mann-Whitney test. (**c**) Intestinal pathology at 48 h after infection. (**d**) Serum concentrations of BUN and CPK (top) and ALT and AST (bottom) at 24 h after infection. *$P < 0.05$ by Mann-Whitney test. Wild-type ($n = 7$); *Nlrc4*$^{-/-}$;*Naip5*$^{-/-}$ ($n = 6$). (**e**) *In vivo* infection of *Casp1*$^{-/-}$ and *Il1b*$^{-/-}$ female mutant mice. Survival was monitored and compared to infected wild-type female mice. Infectious dose was $5 \times 10^8$ live *E. coli* O21:H+. For *Casp1*$^{-/-}$ $P = 0.0025$ and for *Il1b*$^{-/-}$ $P < 0.0001$ by log-rank analysis. Wild type ($n = 9$); *Casp1*$^{-/-}$ ($n = 6$); *Il1b*$^{-/-}$ ($n = 12$). (**f**) Wild-type male and female mice were treated with AVNM for 7 d and then given AVNM plus 5% DSS. At 1, 24 and 48 h after DSS treatment initiation, mice were injected intraperitoneally with 100 μg of antibody to IL-1R (anti–IL-1R) ($n = 13$) or control IgG antibody ($n = 8$), and survival was monitored. $P = 0.0016$ by log-rank analysis (**g**) Wild-type and *Nlrc4*$^{-/-}$;*Naip5*$^{-/-}$ mutant female mice were infected with $5 \times 10^8$ live *E. coli*. At 24 and 48 h after infection, tissues were harvested, homogenized and analyzed for levels of *E. coli* colonization. Data were analyzed by Student's *t* test. Error bars represent s.d. NS, not significant.

Thus, we conclude that mice lacking Naip5-Nlrc4 fare better in response to a systemic *E. coli* O21:H+ infection because they show increased tolerance of the infection[47] rather than because of negative effects on pathogen fitness.

## DISCUSSION

We describe a mouse model of sepsis triggered by dysbiosis and intestinal injury. This model resembles the clinically significant sepsis that is a major problem in human patients undergoing combination therapies involving antibiotics and cytotoxic treatments that damage the gut epithelium[48]. Consistent with the defining features of sepsis, we found that disease in AVNM- plus DSS-treated mice is characterized by systemic dissemination of bacteria, multiple organ damage and systemic responses such as hypothermia. A key conclusion of our study is that sepsis in our AVNM- plus DSS-treated mice is due to inflammasome activation by a specific multidrug-resistant *E. coli* O21:H+ pathobiont. This pathobiont exists in the normal unmanipulated flora but expands dramatically in the gut upon antibiotic treatment and reaches systemic sites following DSS-induced intestinal injury (**Supplementary Fig. 7e**). Systemic inoculation of this pathobiont is sufficient to induce sepsis-like disease.

Similar to our findings, a previous report demonstrated that antibiotic pretreatment rendered mice more susceptible (increased mortality) to DSS treatment[24]. However, this report proposed that disease in AVNM- plus DSS-treated mice is not associated with systemic spread of bacteria but is instead due to a failure to receive appropriate Toll-like receptor–dependent signals needed to repair gut epithelial injury. Consistent with this interpretation, it was found that disease could be alleviated by the oral administration of lipopolysaccharide (LPS), suggesting that Toll-like receptor signaling has a key protective role[24]. In contrast, in our model, mice given AVNM plus DSS showed symptoms of sepsis, not colitis, and oral administration of LPS was not sufficient to prevent sepsis (**Supplementary Fig. 2d**). Indeed, dysbiosis in the AVNM-plus-DSS model may both reduce the levels of beneficial microbes and result in an expansion of pathobionts. The apparent discrepancy between our work and the prior study[24] may be explained by differences in microbial flora in the mice, as well as by methodological differences. For example, the previous study administered drinking water supplemented with 2% DSS to mice for a total of 7 d, which produces milder damage that mice are able to repair. In our model, we used a continuous dose (until the end of the experiment) of 5% DSS. Nevertheless, as systemic infection of normal mice with *E. coli* O21:H+ is sufficient to recapitulate the AVNM-plus-DSS disease model, we favor the hypothesis that pathology in our mice is driven primarily by an *E. coli* pathobiont.

Previous studies have demonstrated a protective role for the inflammasome in mediating pathogen resistance[45,49] and in inducing tissue repair[20,21]. Our study demonstrates that excessive or systemic inflammasome-mediated recognition of a bacterium can lead to pathology and death. In our model, inflammasome activation results in IL-1β–driven immunopathology that the host cannot tolerate (**Supplementary Fig. 7**). The dependence on IL-1β distinguishes our sepsis model from others, including LPS endotoxemia (**Supplementary Fig. 7**)[50–52]. Blockade of IL-1 signaling has long been considered a potential therapeutic treatment for human sepsis, but it has met with little success in clinical trials[53,54]. This is probably because sepsis is a complex disease involving diverse microbes and host immune pathways. Our results highlight the importance of experimentally defining the relevant pathogenic pathways for different microbial causes of sepsis and identifying the causative agents of sepsis in humans.

Antibiotic-resistant *E. coli* present a challenge to antimicrobial medical interventions[4]. Our finding that mice lacking inflammasome components show increased tolerance of systemic *E. coli* infection may have key therapeutic implications. Tolerance (not to be confused with immunological tolerance) is a defense strategy that allows a host to endure an infection without influencing microbial load[47,55]. Tolerance contrasts with resistance, which protects a host by decreasing pathogen burden[47,55]. In contrast to antimicrobials (which work to decrease pathogen burden), therapeutic interventions to increase tolerance could protect the host without imposing negative effects on pathogen fitness. A key benefit of therapeutic modulation of host tolerance pathways is that pathogens are not predicted to evolve antagonistic traits to such therapeutics[47,56,57]. Thus, we propose that the inflammasome may be an alternative therapeutic target for patients showing pathology from antibiotic-resistant pathobionts.

## METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

*Note: Supplementary information is available on the Nature Medicine website.*

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### AUTHOR CONTRIBUTIONS

J.S.A. and R.E.V. conceived of the study, designed experiments and wrote the manuscript. J.S.A. directed the study and performed all experiments. N.J.T. did all mouse intravenous injections and performed the retroviral lethality assay.

### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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# Editing T cell specificity towards leukemia by zinc finger nucleases and lentiviral gene transfer

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**The transfer of high-avidity T cell receptor (TCR) genes isolated from rare tumor-specific lymphocytes into polyclonal T cells is an attractive cancer immunotherapy strategy. However, TCR gene transfer results in competition for surface expression and inappropriate pairing between the exogenous and endogenous TCR chains, resulting in suboptimal activity and potentially harmful unpredicted antigen specificities of the resultant TCRs. We designed zinc-finger nucleases (ZFNs) that promoted the disruption of endogenous TCR β- and α-chain genes. Lymphocytes treated with ZFNs lacked surface expression of CD3-TCR and expanded with the addition of interleukin-7 (IL-7) and IL-15. After lentiviral transfer of a TCR specific for the Wilms tumor 1 (WT1) antigen, these TCR-edited cells expressed the new TCR at high levels, were easily expanded to near purity and were superior at specific antigen recognition compared to donor-matched, unedited TCR-transferred cells. In contrast to unedited TCR-transferred cells, the TCR-edited lymphocytes did not mediate off-target reactivity while maintaining their anti-tumor activity *in vivo*, thus showing that complete editing of T cell specificity generates tumor-specific lymphocytes with improved biosafety profiles.**

Adoptive T cell immunotherapy has shown promise in humans, as seen in the persistent and complete clinical responses observed in patients with leukemia undergoing allogeneic hematopoietic stem cell transplantation followed by the adoptive transfer of donor T lymphocytes[1], as well as in patients with solid cancer after adoptive transfer of autologous tumor-specific cytotoxic T lymphocytes (CTLs)[2,3]. However, several hurdles remain before adoptive immunotherapy can become a broadly applicable therapeutic approach. Tumor-associated antigens are often self antigens, and, thus, lymphocytes with the potential to recognize cancer cells with a sufficiently high avidity are usually deleted or anergized to prevent detrimental autoimmunity. If rare high-avidity tumor-specific lymphocytes can be isolated from a patient with cancer, their expansion to the numbers sufficient for therapy generally requires long-term culture, which is often incompatible with the treatment of rapidly growing tumors and which may result in lymphocyte terminal differentiation and reduced activity.

To overcome these difficulties, the α- and β-chain genes from high-avidity TCRs were cloned from tumor-specific CTLs and genetically inserted into large numbers of human T lymphocytes to impart specificity for autologous tumor cells[4]. Initial clinical studies showed the feasibility and safety of TCR gene transfer, but the therapeutic results have been disappointing compared to those from the infusion of naturally isolated and expanded tumor-specific T cells[5–7]. This probably reflects the limitations of current gene transfer technologies, which often do not establish stable high levels of expression of the exogenous TCR chains and cannot fully redirect T cell specificity. In fact, the tumor-specific α and β TCR chains are added to mature lymphocytes that already express an endogenous TCR. Thus, the transduced cells express four different TCR chains that compete for binding to components of the CD3 complex—the rate-limiting factor controlling the translocation of paired TCR chains to the cell surface—leading to reduced assembly of the tumor-specific TCR at the plasma membrane. Such dual-specific T cells show impaired function, with a lower avidity for targets expressing either the tumor antigen or the ligand for the endogenous TCR. This problem is further exacerbated by the potential for inappropriate pairing of exogenous and endogenous TCR chains, which limits the assembly of proper tumor-specific TCR and leads to the assembly of new TCRs that have not been subjected to tolerance mechanisms and, thus, have unpredictable specificities, including potential autoreactivity[8,9]. Indeed, the risk of severe autoimmune toxicity resulting from the formation of self-reactive TCRs by the mispairing of endogenous

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and exogenous chains has been recently highlighted in a mouse model closely mimicking the clinical setting[10].

Current strategies addressing these hurdles aim to promote the preferential assembly and surface expression[11,12] of the exogenous α and β chains, for example, by using mouse instead of human TCR constant regions, which can enhance homologous pairing but which have proven immunogenic[13]; by incorporating additional cysteines into the exogenous chains to promote homologous pairing and enhance the stability of the exogenous TCR; and/or by codon-usage optimization of the exogenous TCR genes[14]. These strategies increase exogenous TCR expression but fall short of ensuring the high expression levels that are required for high-avidity tumor-specific effectors and, most crucially, do not eliminate mispairing with the endogenously expressed TCR[15].

To overcome these limitations, we report a new strategy based on ZFNs to edit T cell specificity at the DNA level by knocking out the endogenous TCR. ZFNs are engineered endonucleases composed of a tandem array of $C_2H_2$ zinc finger DNA-binding domains, which can be designed to selectively bind a DNA sequence of choice, coupled to the catalytic domain from the type IIS restriction enzyme FokI[16] to introduce a DNA double-strand break (DSB) at a predetermined genomic site. DSBs are predominantly repaired by non-homologous end joining (NHEJ), which is an error-prone process that often introduces nucleotide deletions or insertions (indels) at the site of the break and, consequently, can lead to gene disruption if the ZFN-induced DSB is targeted to a protein-coding sequence[17–20]. Our approach, the TCR gene editing, combines the permanent and heritable somatic knockout of the endogenous TCR genes by transient exposure to ZFNs with the transfer of tumor-specific TCR by state of the art lentiviral vectors. We evaluated TCR gene editing in central memory T ($T_{CM}$) lymphocytes, which are highly clonogenic, and have been shown to have a capacity for self-renewal (and are thus less prone to exhaustion) and enhanced anti-tumor potential[21–24]. We found that the TCR-edited cells had enhanced tumor-killing activity, with sharply reduced non-specific alloreactivity compared to matched cells that underwent conventional TCR gene transfer.

## RESULTS

### Efficient disruption of *TRBC* genes by ZFNs in human T cells

To edit T cell specificity, we first designed a pair of ZFNs (**Supplementary Table 1**) targeting a sequence found within the TCR β chain that is conserved between the β1 and β2 constant-region genes (*TRBC1* and *TRBC2*; **Fig. 1a**) to disrupt the functional TCR β chain gene. We determined the binding specificity of each ZFN using Systematic Evolution of Ligands by Exponential Enrichment (SELEX; **Supplementary Table 2**). We used integrase-defective lentiviral vectors (IDLVs) to transiently express the *TRBC*-targeting ZFNs in lymphocytes (*TRBC*-ZFN IDLVs; **Fig. 1b**)[18,19]. In Jurkat cells, we observed a vector-dose–dependent abrogation of cell-surface expression of the TCR-CD3 complex in up to 20% of the treated cells (**Fig. 1c**). A mismatch-selective endonuclease assay (Cel1 assay) confirmed these results at the genetic level, with up to 26% of the *TRBC* alleles (18% of the *TRBC1* and 8% of the *TRBC2* alleles) being disrupted at the ZFN target site in the treated cells (**Fig. 1d**).

To extend this observation, we activated and sorted T cells from the peripheral blood of healthy donors using beads conjugated with antibodies to CD3 and CD28 (baCD3/CD28) and cultured cells in the presence of IL-7 and IL-15 (5 ng $ml^{-1}$). After 48 h, we exposed the activated T cells to increasing doses of the *TRBC*-ZFN IDLVs for 24 h. We observed abrogation of the cell-surface expression of the TCR-CD3 complex in up to 7% of the treated cells (**Fig. 1e**). A Cel1 analysis of the DNA from cells treated with *TRBC*-ZFN IDLVs cells showed that up to 6% of the *TRBC1* alleles and up to 7% of the *TRBC2* alleles carried mutations at the ZFN target site, which is consistent with the disruption of either allele and the observed loss of CD3 surface expression. In cells sorted for loss of CD3 expression, the frequency of *TRBC1* and *TRBC2* disruption at the DNA level was >30% and >40%, respectively, which is more than the minimal expected level of 25% each, considering that only one out of four alleles is expressed in each cell and that disruption of that one allele is sufficient to abrogate CD3 surface expression (**Fig. 1f**). Amplification and sequencing of the *TRBC1* and *TRBC2* DNA from treated lymphocytes confirmed the occurrence of indels at the ZFN target site, resulting in the disruption of the targeted genes (**Fig. 1g**).

### Expansion and purification of *TRBC*-disrupted T lymphocytes

Because memory T lymphocytes are less dependent on TCR signals for homeostatic proliferation than are naive T cells[25], we investigated whether homeostatic cytokines could promote the survival and growth of previously activated cells in the absence of TCR expression and CD3 surface expression ($CD3^-$ cells). Notably, the *TRBC*-ZFN–treated cells expanded in cultures supplemented with low-dose IL-7 and IL-15 (**Fig. 2a**), with the proportion of $CD3^-$ cells remaining stable for more than 50 d in the absence of TCR triggering. In the absence of cytokines, both $CD3^-$ and $CD3^+$ cells died within 7 d (data not shown), indicating that homeostatic cytokines are required for T cell survival. Thus ZFN exposure was well tolerated in primary human lymphocytes and resulted in the stable disruption of the targeted *TRBC* gene. $CD3^-$ cells did not proliferate in response to the TCR-dependent mitogen phytohaemagglutinin (PHA) and were rapidly overgrown by the responding $CD3^+$ lymphocytes (**Fig. 2b**).

We found no phenotypic differences between $CD3^+$ and $CD3^-$ lymphocytes, which both had similar CD4:CD8 ratios and seemed to acquire $T_{CM}$ surface phenotypes in culture, as evidenced by high expression of CD62L, CD27, CD28 and IL-7 receptor α (IL-7Rα) (**Fig. 2c**). Sorted $CD3^-$ cells (**Fig. 2d**) expanded in response to IL-7 and IL-15 for more than 3 weeks with growth rates similar to those of $CD3^+$ cells (**Fig. 2e**), confirming in a pure population of $CD3^-$ cells that homeostatic cytokines do not require TCR signaling to promote the survival and proliferation of previously activated cells. These data show the successful generation of a new population of T lymphocytes that have the phenotypic characteristics of $T_{CM}$ lymphocytes but that have permanently disrupted expression of TCR.

### TCR-β editing results in high-avidity tumor-specific T cells

We next assessed whether the endogenous TCR-deficient lymphocytes could be further manipulated to generate tumor specific T cells. We selected a TCR specific for the WT1 antigen, which is a therapeutically relevant antigen that contributes to oncogenesis and is broadly overexpressed by leukemias and many solid tumors. We used codon-optimized, human leukocyte antigen A2 (HLA-A2)–restricted TCR α- and β-chain complementary DNAs specific for the $WT1_{126–134}$ peptide (WT1-TCR) that had been modified to incorporate matched cysteines in the constant regions to facilitate homologous pairing and high levels of expression[26]. To promote robust and coordinate expression of the α and β TCR chains in transduced lymphocytes, we generated integrating lentiviral vectors encoding both chains under a bidirectional promoter derived from the human phosphoglycerate kinase (PGK) gene (**Fig. 3a**)[27].

We first treated T lymphocytes with *TRBC*-ZFN IDLVs to disrupt the endogenous *TRBC* gene. We sorted the resulting CD3⁻ cells and successfully transduced them (49.5% ± 30% (mean ± s.d.) transduction efficiency, $n = 4$ transductions) with WT1-TCR lentiviral vector, indicating that these cells were permissive to further genetic manipulation. Expression of the transferred WT1-TCR rescued the surface translocation of CD3, which was coexpressed in balanced proportion with the WT1-TCR Vβ21 chain in these TCR-β–edited cells (**Fig. 3b**). In contrast to the matched unedited TCR-transferred lymphocytes (**Fig. 3b**), TCR-β–edited cells expressing WT1-TCR could be enriched to >90% purity using polyclonal stimulation, indicating that surface expression of the transferred TCR-CD3 complex was necessary and sufficient to promote TCR-mediated expansion (compare to **Fig. 2b**). The exogenous Vβ21 chain was expressed in TCR-β–edited lymphocytes at up to twofold higher mean levels than in the unedited TCR-transferred cells and reached similar expression levels as those of the endogenous Vβ21 chain from control T cells (**Fig. 3c**). The expression of the Vβ21 chain was stably maintained in culture (**Fig. 3d**). Accordingly, after transduction with the same dose of WT1-TCR lentiviral vector, up to 22% of the TCR-β–edited lymphocytes bound the $WT1_{126-134}$ pentamer, as compared to only 2.6% of the unedited TCR-transferred cells (**Fig. 3c**). Thus, in the absence of competition from

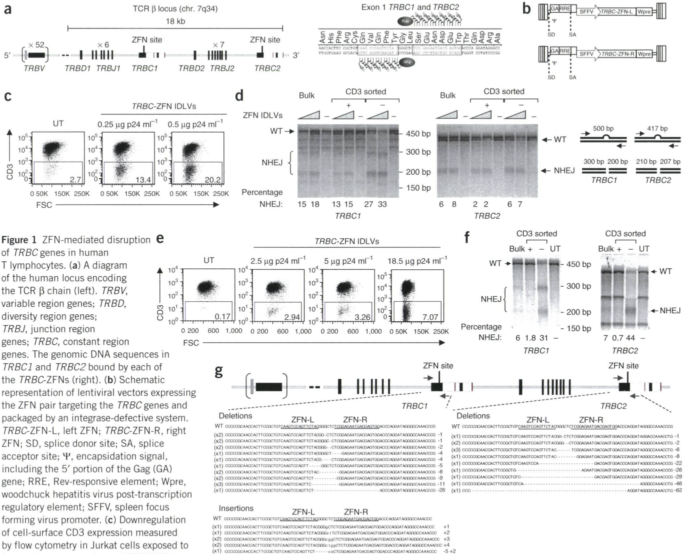


**Figure 1** ZFN-mediated disruption of *TRBC* genes in human T lymphocytes. (**a**) A diagram of the human locus encoding the TCR β chain (left). *TRBV*, variable region genes; *TRBD*, diversity region genes; *TRBJ*, junction region genes; *TRBC*, constant region genes. The genomic DNA sequences in *TRBC1* and *TRBC2* bound by each of the *TRBC*-ZFNs (right). (**b**) Schematic representation of lentiviral vectors expressing the ZFN pair targeting the *TRBC* genes and packaged by an integrase-defective system. *TRBC*-ZFN-L, left ZFN; *TRBC*-ZFN-R, right ZFN; SD, splice donor site; SA, splice acceptor site; Ψ, encapsidation signal, including the 5′ portion of the Gag (GA) gene; RRE, Rev-responsive element; Wpre, woodchuck hepatitis virus post-transcription regulatory element; SFFV, spleen focus forming virus promoter. (**c**) Downregulation of cell-surface CD3 expression measured by flow cytometry in Jurkat cells exposed to increasing concentrations of *TRBC*-ZFN IDLVs (measured by human immunodeficiency virus (HIV) Gag protein p24 equivalents). The percentage of CD3⁻ cells is indicated in the quadrants. UT, untransduced cells; FSC, forward scatter. (**d**) Amount of targeted gene disruption measured by a mismatch-selective Cel1 endonuclease assay on DNA amplified from the cells shown in **c** before (bulk, unsorted (*TRBC*-ZFN IDLVs)) and after sorting for the presence (+ in the top of the gel) or absence (– in the top of the gel) of CD3 on the cell surface. The schematic on the right shows the expected size of the intact amplicon (upper wild-type (WT) bands in the gel) and the Cel1 cleavage products of heteroduplexes carrying mismatches around the ZFN target site introduced by NHEJ (lower bands in the gel; the two cleavage products comigrate at ~200 bp for *TRBC2*). The other bands are nonspecific amplification products. The calculated amount of targeted gene disruption in *TRBC1* and *TRBC2* is shown at the bottom. (**e**,**f**) Downregulation of cell-surface CD3 expression (**e**) and the amount of targeted gene disruption (**f**) in primary human lymphocytes exposed to increasing concentrations of *TRBC*-ZFN IDLVs after stimulation with baCD3/CD28 and cultured with IL-7 and IL-15. The other bands in the *TRBC2* gel derive from a single nucleotide polymorphism in that locus. The percentage of CD3⁻ cells is indicated in the quadrants. In the top of the gel, bulk indicates unsorted cells, and + and – indicate cells sorted for presence or absence of CD3 on the cell surface, respectively. (**g**) The *TRBC1* and *TRBC2* DNA in ZFN-treated lymphocytes amplified, cloned and sequenced to confirm ZFN cleavage. Sequence alignment revealed several nucleotide indels encompassing the ZFN target region. The left column indicates the number of clones retrieved, and the right column indicates the number of nucleotides deleted or inserted.

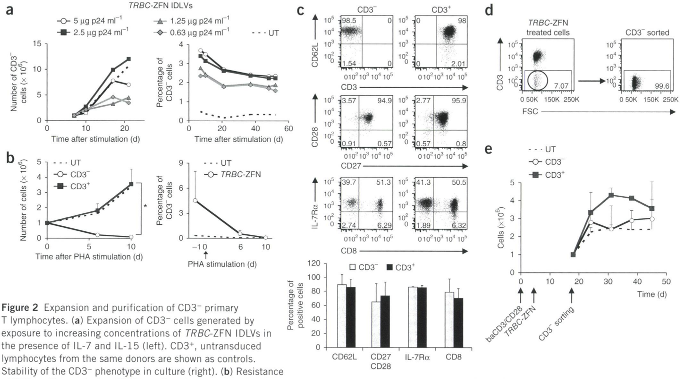


**Figure 2** Expansion and purification of CD3− primary T lymphocytes. (**a**) Expansion of CD3− cells generated by exposure to increasing concentrations of *TRBC*-ZFN IDLVs in the presence of IL-7 and IL-15 (left). CD3+, untransduced lymphocytes from the same donors are shown as controls. Stability of the CD3− phenotype in culture (right). (**b**) Resistance of CD3− cells to polyclonal TCR-mediated stimulation. Shown are results from lymphocytes activated with PHA after exposure to *TRBC*-ZFN IDLVs and cultured with IL-7, IL-15 and IL-2 (left). Data are means + s.d. $n = 2$. $*P < 0.05$ by Mann-Whitney test. Also shown are the average + s.d. of the percentage of CD3− lymphocytes before and after PHA stimulation (right). (**c**) Representative plots (top) and average histograms (bottom) showing the expression of CD62L, CD28, CD27 and IL-7Rα in CD3− and CD3+ gated cells. The percentages of events are indicated in each quadrant. Data are mean + s.d. (**d**,**e**) Sorting of CD3− cells to near homogeneity (**d**) and expansion of sorted CD3− cells (**e**) compared to that of CD3+ cells and untransduced lymphocytes in the presence of IL-7 and IL-15. The percentage of CD3− cells is indicated in the quadrants. Data are mean + s.d.

the endogenous TCR β chain, the surface expression of the transgenic TCR β chain reached physiological levels. TCR-β–edited cells killed $WT1_{126-134}$ peptide-pulsed HLA-A2+ targets more effectively than did unedited cells (**Fig. 3e**; half maximal effective concentration ($EC_{50}$): TCR-β–edited cells, 90.45 nM, 95% CI 58.47–139.9 nM; TCR-transferred cells, 312.3 nM, 95% CI 235–415 nM), probably reflecting the higher frequency and expression of the transgenic WT1-TCR in the TCR-edited T cells and showing the functional advantage offered by the expression of a tumor-specific exogenous TCR in a host CTL with abrogated endogenous TCR β-chain expression.

Theoretically, surface re-expression of the endogenous TCR α chain may still occur in TCR-β–edited cells after TCR gene transfer. To directly assess the potential for residual mispairing in TCR β-chain–disrupted lymphocytes, we transduced CD3− cells using a lentiviral vector encoding only the WT1-specific TCR β-chain gene and the truncated low-affinity nerve growth factor receptor (ΔLNGFR) marker (WT1–TCR-β/ΔLNGFR-LV). Despite the absence of a WT1-specific α chain, which is required for correct WT1-TCR pairing, we detected expression of the Vβ21 chain in up to 84% of ΔLNGFR+ *TRBC*-disrupted cells (**Fig. 3f**). This result shows that even a cysteine-modified TCR β chain, which is required to complex with an α chain for surface expression, can efficiently mispair with the endogenous TCR α chain when expressed in a cell with endogenous *TRBC* disruption.

## Full TCR-α/β gene editing results in high-avidity T cells

To eliminate the potential for TCR chain mispairing, we designed another pair of ZFNs targeting the constant region of the TCR α chain (*TRAC*) gene (**Fig. 4a**) and obtained TCR-α–edited T lymphocytes using the same protocol described above for TCR-β editing (**Supplementary Fig. 1**). We then developed a strategy for complete editing of both TCR-α and TCR-β (TCR-α/β editing) that permitted the rapid isolation of the engineered cells at each sequential step of TCR chain disruption or replacement. This strategy made use of a lentiviral vector to stably express either the α or β WT1-specific TCR chain and used IDLVs or adenoviral vectors (AdVs) to transiently express *TRBC*- or *TRAC*-targeting ZFNs (*TRBC*-ZFN AdV or *TRAC*-ZFN AdV) in lymphocytes. Whereas CD3− cells were efficiently generated with both of the ZFN-expressing vectors we tested (**Supplementary Fig. 2**), using AdVs was more efficient, and, therefore, we selected the AdVs for the purpose of complete TCR editing (**Fig. 4b**). After AdV transduction, ZFN activity was transient in the treated cells and was detectable only for the first 2–3 d after infection, peaking at 24 h after infection (**Supplementary Fig. 3**).

For the TCR-α/β editing, we first exposed cells harvested from healthy donors to *TRAC*-ZFN AdV after activation with baCD3/CD28 and culture in the presence of IL-7 and IL-15, and the resulting CD3− cells (36% ± 13% CD3− cells (mean ± s.d.)) were sorted 5–8 d after ZFN transduction, when surface protein turnover allows for discriminating CD3− cells and ZFN activity is no longer detectable because of vector dilution during cell proliferation. We transduced CD3− cells (65% ± 31% (mean ± s.d.) transduction efficiency) with a lentiviral vector encoding the WT1-TCR α chain under the PGK promoter (WT1-TCR α chain lentiviral vector). We then sorted the cells with rescued CD3 expression, stimulated them with baCD3/CD28 and exposed them to *TRBC*-ZFN AdV. The second round of ZFN exposure yielded 19% ± 8% (mean ± s.d.) newly CD3$^-$ cells, indicating that primary T lymphocytes are permissive to multiple rounds of ZFN treatment. We sorted the CD3$^-$ cells and transduced them (25% ± 15% (mean ± s.d.) transduction efficiency, $n = 4$ experiments) with a WT1-TCR β chain lentiviral vector. Expression of the transferred WT1 β chain again rescued the surface translocation of CD3, which was coexpressed in balanced proportion with the WT1-TCR β chain in the TCR-edited cells (**Fig. 4b**). TCR-α/β–edited cells could be enriched to near purity by polyclonal stimulation after TCR gene transfer (**Fig. 4b**) and homogenously expressed the high levels of the WT1-specific TCR that are required to bind the WT1$_{126-134}$ HLA-A2 pentamer (**Fig. 4c**). These results indicate that surface expression of the transferred TCR-CD3 complex in cells edited for both TCR-α and TCR-β is necessary and sufficient to promote the expansion of the cells with the desired antigen specificity.

We confirmed the concomitant disruption of the α and β endogenous TCR chains in cells edited for both TCR-α and TCR-β using a Cel1 assay (**Supplementary Fig. 4**). We used the consensus DNA-binding site experimentally determined using SELEX for the *TRBC*- and *TRAC*-ZFNs for the bioinformatic prediction of the 15 most likely

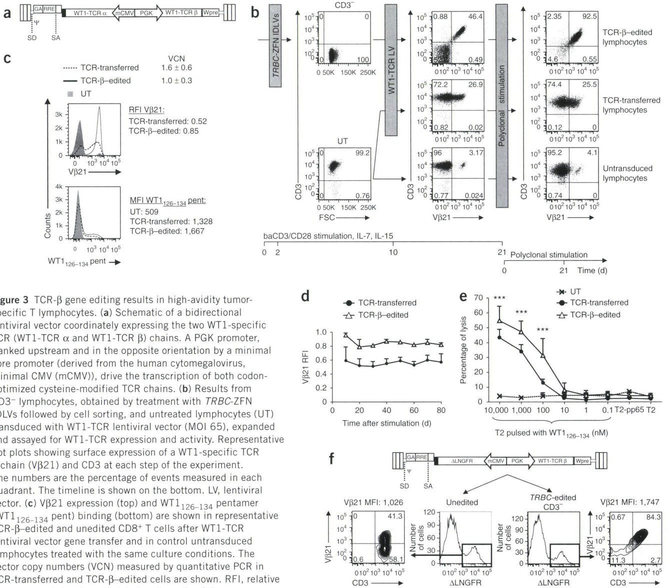


**Figure 3** TCR-β gene editing results in high-avidity tumor-specific T lymphocytes. (**a**) Schematic of a bidirectional lentiviral vector coordinately expressing the two WT1-specific TCR (WT1-TCR α and WT1-TCR β) chains. A PGK promoter, flanked upstream and in the opposite orientation by a minimal core promoter (derived from the human cytomegalovirus, minimal CMV (mCMV)), drive the transcription of both codon-optimized cysteine-modified TCR chains. (**b**) Results from CD3$^-$ lymphocytes, obtained by treatment with *TRBC*-ZFN IDLVs followed by cell sorting, and untreated lymphocytes (UT) transduced with WT1-TCR lentiviral vector (MOI 65), expanded and assayed for WT1-TCR expression and activity. Representative dot plots showing surface expression of a WT1-specific TCR β chain (Vβ21) and CD3 at each step of the experiment. The numbers are the percentage of events measured in each quadrant. The timeline is shown on the bottom. LV, lentiviral vector. (**c**) Vβ21 expression (top) and WT1$_{126-134}$ pentamer (WT1$_{126-134}$ pent) binding (bottom) are shown in representative TCR-β–edited and unedited CD8$^+$ T cells after WT1-TCR lentiviral vector gene transfer and in control untransduced lymphocytes treated with the same culture conditions. The vector copy numbers (VCN) measured by quantitative PCR in TCR-transferred and TCR-β–edited cells are shown. RFI, relative fluorescence intensity calculated as the ratio of the mean fluorescence intensity (MFI) of Vβ21 measured in genetically modified lymphocytes to the MFI of Vβ21 in naturally expressing T cells. (**d**) Stability of the surface expression of the Vβ21 TCR chain. (**e**) Cytotoxic assay with TCR-β–edited and TCR-transferred cells. Shown is the functional activity measured by a $^{51}$chromium release assay for the lysis of labeled T2 cells pulsed with increasing concentrations of the WT1$_{126-134}$ HLA-A2 restricted peptide or with the irrelevant CMV-derived pp65$_{495-503}$ HLA-A2 restricted peptide as negative control. Data are mean + s.d. of the percentage of lysis at an effector-to-target ratio of 12. ***$P < 0.001$ by two-way analysis of variance test (ANOVA) plus Bonferroni post test. $n = 6$. (**f**) Mispairing of cysteine-modified TCR β chain and endogenous α chain. CD3$^-$ cells sorted from *TRBC*-disrupted lymphocytes (right) and unedited cells (left) transduced with a bidirectional lentiviral vector encoding the β chain of the WT1-specific TCR and the truncated low-affinity nerve growth factor receptor (ΔLNGFR) complementary DNA under the control of the bidirectional PGK promoter. Transduction efficiency was assessed as percentage of ΔLNGFR$^+$ lymphocytes (histograms). Vβ21 expression was measured on ΔLNGFR$^+$ cells (plots). The MFI of Vβ21 is shown. Results from one representative experiment of six total experiments are shown. The numbers are the percentage of events measured in each quadrant.

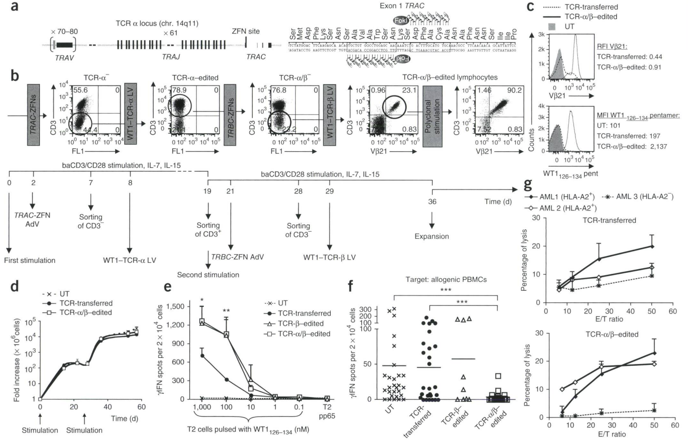


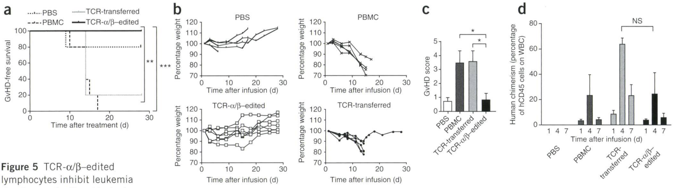
**Figure 4** Full gene editing of TCR-α and TCR-β results in high-avidity tumor-specific lymphocytes with sharply reduced alloreactivity. (**a**) A diagram of the human locus encoding the TCR α chain (left) and the genomic DNA sequences in *TRAC* bound by each *TRAC*-ZFN (right). (**b**) Schematic, timeline and representative flow cytometry analysis at each step of a protocol optimized for full TCR gene editing. Surface expression of CD3 and Vβ21 is shown, with the numbers indicating the percentage of events measured in each quadrant. (**c**) Vβ21 TCR expression (top) and $\mathrm{WT1}_{126-134}$ pentamer binding (bottom) are shown in CD8⁺ T cells with the TCR-α and TCR-β chains disrupted and transduced with the WT1-TCR chains (TCR-α/β–edited), in unedited WT1-TCR lentiviral vector transduced cells (TCR-transferred) and in untransduced lymphocytes treated with the same culture conditions. The Vβ21 RFI and $\mathrm{WT1}_{126-134}$ pentamer MFI are indicated on the right for cells gated on Vβ21 and CD8. (**d**) The fold increase in the number of TCR-transferred and TCR-α/β–edited T lymphocytes activated with antibody to CD3, irradiated allogeneic peripheral blood mononuclear cells (PBMCs), irradiated allogeneic EBV cell lines and IL-2. Every step of activation is indicated by an arrow. (**e**–**g**) Functional activity of genetically modified lymphocytes. Shown are results from TCR-α/β–edited, TCR-β–edited, TCR-transferred (sorted for high expression of Vβ21) and untransduced lymphocytes tested by γ interferon (γIFN) ELISpot assay against T2 cells pulsed with increasing concentrations of the $\mathrm{WT1}_{126-134}$ HLA-A2 restricted peptide or the irrelevant CMV-derived $\mathrm{pp65}_{495-503}$ HLA-A2 restricted peptide as negative control (**e**) or irradiated allogeneic PBMCs (**f**). Specific spots are shown on the *y* axis as spots produced in the presence of stimulators minus the spots produced by the effectors alone. Mean + s.d. are shown in **e**; the horizontal lines in **f** represent the mean of each group of values. (**g**) TCR-α/β–edited and Vβ21-sorted TCR-transferred cells challenged with HLA-A2⁺ and HLA-A2⁻ AML primary blasts, harvested from patients with leukemia, in a cytotoxic assay. E/T ratio, effector-to-target ratio. Mean + s.d. are shown. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by two-way ANOVA plus Bonferroni post test (**e**) and by Mann-Whitney test (**f**).

off-target sites for each ZFN set in the human genome. Cel1 assays performed on T lymphocytes edited for TCR-α only, TCR-β only or both TCR-α and TCR-β revealed no detectable signs of NHEJ (1% limit of detection) at any of these 30 genomic sites, suggesting a high rate of target specificity for both of the ZFN sets (**Supplementary Figs. 5** and **6**). We found no phenotypic differences in the TCR-transferred and the TCR-α/β–edited lymphocytes, which had a preferential $T_{CM}$ surface phenotype (**Supplementary Fig. 7a**) and, after stimulation in the absence of co-stimulation, expanded and differentiated with similar kinetics (**Fig. 4d** and **Supplementary Fig. 7b**).

## TCR-α/β–edited cells are functional and highly tumor specific

To verify their function and avidity, we compared cells edited for both TCR-α and TCR-β and those edited for TCR-β only to unedited cells, which we transduced with the WT1-TCR lentiviral vector and which we selected for high transgene expression, for the ability to recognize HLA-A2⁺ and HLA-A2⁻ WT1⁺ targets. Similar to the cells edited for TCR-β only, TCR-α/β–edited lymphocytes were superior to the TCR-transferred lymphocytes at recognizing specific WT1⁺ HLA-A2⁺ targets (**Fig. 4e**). Full editing of both TCR-α and TCR-β was required to completely abrogate off-target reactivity (**Fig. 4f**). We confirmed these results on clones that could be isolated and expanded with similar efficiencies from TCR-α/β–edited cells and from TCR-transferred cells, indicating that the editing process does not impair the expansion or responsiveness of T lymphocytes (**Supplementary Fig. 8**). We confirmed the high specificity and lytic capacity of TCR-α/β–edited cells by challenge with HLA-A2⁺ and HLA-A2⁻ WT1⁺ acute myeloid leukemia (AML) blasts harvested from patients (**Fig. 4g**).

To confirm that the TCR-α and TCR-β editing process abrogated the off-target reactivity of the gene-modified cells, we infused



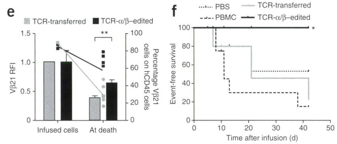


**Figure 5** TCR-α/β–edited lymphocytes inhibit leukemia development without inducing GvHD in immunodeficient mice. (**a**,**b**) Survival without severe GvHD (**a**) and weight loss (**b**) in mice after infusion of PBS ($n = 5$), PBMCs ($n = 6$), TCR-transferred cells sorted for Vβ21 expression ($n = 5$) or matched TCR-α/β–edited lymphocytes ($n = 7$). (**c**) The GvHD scores of each group of mice. Mean + s.d. are shown. (**d**) Human chimerism in each group of mice. hCD45, human CD45; WBC, white blood cells; NS, not significant. Mean + s.d. are shown. (**e**) Expression of WT1-specific TCR quantified before infusion and at death on gated human cells (hCD45$^+$) and expressed as a percentage of Vβ21$^+$ cells (lines) and Vβ21 RFI (columns). TCR-transferred cells are shown in gray and TCR-α/β–edited cells are shown in black. Mean + s.d. are shown. (**f**) Event-free survival (free of GvHD or leukemia) in NSG mice after infusion of AML blasts from HLA-A2$^+$ patients plus PBS ($n = 11$), untransduced lymphocytes ($n = 8$), TCR-transferred cells sorted for Vβ21 expression ($n = 10$) or TCR-α/β–edited lymphocytes ($n = 8$), all from the same donors. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by log-rank Mantel-Cox test (**a**,**f**) and by Mann-Whitney test (**c**–**e**).

donor-matched TCR-transferred, TCR-α/β–edited or unmanipulated cells into 10- to 12-week-old nonobese diabetic severe combined immunodeficient γ chain knockout (NSG) mice that had been conditioned with 175 cGy irradiation. Six of the six mice receiving unmanipulated T cells and four of the five mice infused with TCR-transferred lymphocytes developed lethal graft-versus-host disease (GvHD) within 2 weeks after the infusion, whereas none of the mice receiving TCR-α/β–edited cells ($n = 7$) developed GvHD in the 4 weeks of follow up after infusion (**Fig. 5a**–**c**). All experimental groups showed substantial and similar engraftment rates (**Fig. 5d**). Notably, despite the fact that gene-modified cells homogenously expressed high levels of the WT1-specific TCR before infusion, expression of the transgenic TCR was significantly lower in TCR-transferred cells than in TCR-α/β–edited lymphocytes harvested from the mice (**Fig. 5e**). This observation suggests a selective *in vivo* expansion of T cells expressing alloreactive TCR pairs, rather than transgenic tumor-specific TCR, in TCR-transferred cells, a condition that was prevented by abrogating endogenous TCR expression by the editing process.

Finally, for a comprehensive assessment of the safety and antitumor efficacy of cells edited for both TCR-α and TCR-β, we infused matched TCR-transferred, TCR-α/β–edited or unmanipulated cells into 10- to 12-week-old NSG mice conditioned with 175 cGy irradiation and infused with WT1$^+$ leukemic blasts harvested from two HLA-A2$^+$ patients with AML. In this model, mice are expected to die either from GvHD or leukemia. Event-free survival, defined as survival in the absence of GvHD and leukemia, was superior in the cohort of mice receiving TCR-α/β–edited cells than in mice infused with TCR-transferred cells ($P < 0.05$) or those receiving unmanipulated lymphocytes ($P < 0.01$; **Fig. 5f**). The anti-tumor activity was dependent on WT1-TCR expression and was significant in mice treated with TCR-transferred cells ($P < 0.05$) and those treated with TCR-α/β–edited cells ($P < 0.05$) when compared to untreated mice and mice treated with untransduced lymphocytes. However, mice infused with unedited TCR-transferred cells suffered rapid-onset and lethal GvHD (**Fig. 5a**). Indeed, the observed mortality in mice receiving TCR-transferred cells was caused entirely by GvHD, whereas 50% and 100% of the deaths in mice that received unmanipulated lymphocytes or PBS, respectively, were caused by leukemia growth. These results indicate the advantage of cells edited for both TCR-α and TCR-β, compared to TCR-transferred cells, in mediating a safe anti-leukemia response *in vivo*.

## DISCUSSION

Suboptimal activity toward their specific target and the generation of potentially harmful unpredicted specificities are common drawbacks of TCR gene transfer in human T cells[9]. Our results show that combining ZFN-driven disruption of the endogenous TCR α- and β-chain genes with lentiviral delivery of a tumor-specific TCR results in the successful and complete editing of primary CD8$^+$ T cell specificity. Because both disruption and replacement are performed at the genetic level, edited TCR specificity is stable and is transmitted to cell progeny. The absence of the endogenous TCR chains on TCR-edited cells allows for a more robust and homogenous expression of the introduced tumor-specific TCR, resulting in a higher avidity for the specific target, and completely overcomes the safety issues raised by TCR mispairing.

The advantage of these edited cells is best exemplified by the much higher fraction of $WT1_{126-134}$ pentamer binding cells observed in the edited cells compared to that found in unedited TCR-transferred lymphocytes. Accordingly, TCR-α/β–edited cells showed a higher activity than TCR-transferred cells against relevant targets, including blasts harvested from patients with AML. Most notably, the cells edited for both TCR-α and TCR-β had sharply reduced activity against nonspecific targets, further emphasizing the utility of preventing the formation of mispaired TCRs and suggesting that such TCR-edited cells may possess improved safety with respect to the risk of inducing autoimmune diseases. In a mouse preclinical model, the mispairing of endogenous and exogenous TCR chains has been clearly linked to severe toxicity[10]. Although molecular modifications of the exogenous TCR genes can reduce the extent of this mispairing, strategies that are able to completely abrogate this risk have not been previously reported. Recently, the expression of siRNA has been proposed to specifically downregulate endogenous TCR expression. However, this approach reduced, but did not eliminate, the expression of the endogenous TCR[12]. Here we show that even cysteine modification of the TCR chains, one of the most promising approaches to address mispairing[26,28], is not sufficient to abrogate this unwanted effect. The strategy we describe here allows for the rapid generation of large numbers of tumor-specific T cells expressing high functional levels of only the desired TCR, thus eliminating the risk of TCR mispairing. In a humanized mouse model, we found no GvHD after infusion of TCR-$\alpha/\beta$–edited lymphocytes whereas the infusion of TCR-transferred lymphocytes induced lethal GvHD within 2 weeks in the majority of infused mice, showing *in vivo* that forced expression in human T cells of an exogenous TCR *per se* does not prevent alloreactivity, even when the TCR chains include cysteine modifications.

Several sequential steps of genetic manipulation ensure complete editing of both TCR-$\alpha$ and TCR-$\beta$ in the final cellular product. Here, disruption of each TCR chain gene by ZFNs was accomplished separately because (i) the simultaneous expression of four individual ZFNs (two sets) targeting distinct genomic sites had the potential to lead to unwanted ZFN dimer pairings and, therefore, increased off-target activity, at least with some ZFNs[29], and (ii) identification of the gene-targeted cells took advantage of the dependence of the surface expression of CD3 on the expression of both TCR chains. Surface CD3 expression thus provides a convenient surrogate marker to rapidly select viable cells with the desired gene modification at each disruption or replacement step of the protocol. This approach allows the whole process to be completed within a culture time that is shorter than that reported in some settings to isolate and expand viral-specific T cells[30] and to generate a pure population of antigen-specific T cells in the absence of antigenic stimulation.

To our knowledge, this is the first time that a double genetic disruption that is efficiently followed by gene replacement has been achieved in primary human cells. Notably, the gene editing did not detectably affect the phenotype, function or proliferative potential of the engineered cells. TCR-edited lymphocytes circulating 4 weeks after infusion continued to express high levels of the tumor-specific TCR, suggesting the potential long-term stability of such cells *in vivo*. Indeed, when challenged *in vivo* with primary human leukemias, TCR-edited cells showed substantial anti-tumor efficacy and, because they were devoid of off-target reactivity, were superior to TCR-transferred lymphocytes in promoting event-free survival. Further advances in humanization of mouse models, possibly including the *in vivo* production of human cytokines, may allow for the assessment of the TCR-editing protocol for long-term T cell viability. Clinical trials based on ZFN-mediated disruption of the *CCR5* (clinicaltrials.gov identification numbers NCT 00842634 and NCT 01044654) and glucocorticoid receptor (NCT 01082926) genes in T lymphocytes are ongoing, indicating the feasibility of pursuing a ZFN-based therapy for clinical applications[17]. Clinical development of our strategy could thus proceed using well-established protocols for producing genetically engineered T cells for use in humans, and the application of this new approach to modify distinct T cell subpopulations may substantially enhance the therapeutic potential and reduce the possible risk of toxicity of immunotherapy for a variety of cancers.

## METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

*Note: Supplementary information is available on the Nature Medicine website.*

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### AUTHOR CONTRIBUTIONS

E.P. and P.G. designed experiments, performed research, analyzed data and wrote the manuscript. A.L. designed research and analyzed data. Z.M. performed research and analyzed data. P.-Q.L., A.R. and V.C. designed and were responsible for assembly of the *TRBC*-ZFN. D.E.P. and L.Z. designed and were responsible for assembly of the *TRAC*-ZFN. J.K. optimized the WT1-TCR genes. B.C. performed *in vivo* experiments. A.B. set up the protocols of the T cell culture and assisted with experimental design. M.P. supervised the histological analyses. G.C., F.C., C. Bordignon, P.D. Greenberg, M.C.H. and P.D. Gregory assisted with experimental design and revised the paper. L.N. designed the research, analyzed the data and wrote the manuscript. C. Bonini designed the research, analyzed the data, wrote the manuscript and acted as senior author of the study.

### COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemedicine/.

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# MRSA epidemic linked to a quickly spreading colonization and virulence determinant

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**The molecular processes underlying epidemic waves of methicillin-resistant *Staphylococcus aureus* (MRSA) infection are poorly understood[1]. Although a major role has been attributed to the acquisition of virulence determinants by horizontal gene transfer[2], there are insufficient epidemiological and functional data supporting that concept. We here report the spread of clones containing a previously extremely rare[3,4] mobile genetic element–encoded gene, *sasX*. We demonstrate that *sasX* has a key role in MRSA colonization and pathogenesis, substantially enhancing nasal colonization, lung disease and abscess formation and promoting mechanisms of immune evasion. Moreover, we observed the recent spread of *sasX* from sequence type 239 (ST239) to invasive clones belonging to other sequence types. Our study identifies *sasX* as a quickly spreading crucial determinant of MRSA pathogenic success and a promising target for therapeutic interference. Our results provide proof of principle that horizontal gene transfer of key virulence determinants drives MRSA epidemic waves.**

*S. aureus* is a dangerous pathogen, causing a multitude of serious and sometimes life-threatening diseases around the globe. This is mostly due to widespread antibiotic resistance among *S. aureus* isolates, which considerably complicates the treatment of *S. aureus* infections[5]. MRSA strains in particular represent a major problem for public health systems, as they combine resistance to methicillin and other β-lactam antibiotics with relatively minor fitness costs, resulting in high infiltration of hospital and community settings[6]. Remarkably, in the US, the estimated number of deaths due to MRSA infections exceeds that due to HIV/AIDS[7]. Although molecular typing and genome sequencing have provided better insight into MRSA epidemiology in recent years[8], there is a severe lack of knowledge regarding the mechanisms by which specific molecular determinants cause the spread and pathogenic success of MRSA clones.

Only a very limited number of MRSA clones, belonging to five clonal complexes, are responsible for the majority of MRSA infections worldwide[1]. Specific sequence types are characteristic for a given geographical location. In China, as in most Asian countries, ST239 is predominant[9]. Recently, genome sequencing of an ST239 strain revealed a new gene of unknown function (SATW20_21850) that is present in only three of the 43 global ST239 strains that have been sequenced[3,4,10] and absent from all other known *S. aureus* genomes[3]. On the basis of its amino acid sequence, which contains a signal peptide and an LPXTG surface-anchoring motif, this gene encodes a secreted, surface-anchored protein of 15 kDa in its processed form. It is located at the 3′ end region of a 127.2-kb ΦSPβ-like prophage, which lacks other virulence-associated genes[3]. There is no similarity of the surface protein to other proteins found in the NCBI databases except for the highly similar, ΦSPβ-encoded SesI of *S. epidermidis* RP62A[11]. We termed the gene *sasX* and showed that it is absent from other major global MRSA strains by analyzing a series of strains from divergent clonal and geographical backgrounds (**Supplementary Data**).

Prompted by the epidemiological connection of *sasX* to MRSA ST239, we wanted to evaluate the hypothesis that *sasX* represents a key factor determining the spread of MRSA in China[9]. To that end, we determined sequence type distribution and frequency of *sasX*-positive clones among >800 randomly selected *S. aureus* isolates from patients with *S. aureus* infections at three large teaching hospitals located in eastern China between 2003 and 2011 (**Fig. 1a**). The situation in China in terms of MRSA infection rate and the dominance of the ST239 (Brazil/Hungary) clone is largely characteristic of that in the Asian and Australian region, except Japan, South Korea and Australia[9,12–15]. Accordingly, ST239 clones were also predominant among the isolates investigated in our study, with ST5 being the second most frequently isolated sequence type (**Supplementary Fig. 1**). Notably, the frequency of *sasX*-positive invasive *S. aureus* clones increased significantly from 2003 to 2011 (from 19% to 31%, $P = 0.0026$). Among MRSA isolates, the frequency of *sasX* increased from 21% to 39% ($P = 0.0028$), whereas the increase among methicillin-sensitive *S. aureus* (MSSA) was not significant (**Fig. 1b**). Furthermore, *sasX* was found almost exclusively among ST239 strains in 2003–2005, whereas its frequency among isolates of several other sequence types considerably increased since then (increase from 5% to 28% of non-ST239

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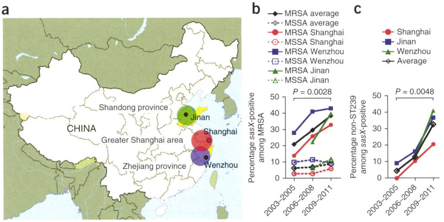

**Figure 1** Spread of *sasX*-positive clones in China. (**a**) A total of 807 isolates from three teaching hospitals in eastern China were analyzed for sequence type, methicillin resistance and presence of *sasX*. (**b**) Percentage of *sasX*-positive among MSSA and MRSA isolates. Statistical analysis is by $\chi^2$ test of all MRSA isolates (average curve, shown in black). The *P* value including all (MRSA and MSSA) isolates is 0.0026. (**c**) Percentage of *sasX*-positive isolates in sequence types other than ST239. Statistical analysis is by Fisher's exact test of all isolates (average curve, shown in black).

clones among *sasX*-positive *S. aureus* between 2003–2005 and 2009–2011, $P = 0.0048$; **Fig. 1c**). Moreover, *sasX* was extremely rare (only one positive clone among 169 tested) among a collection of community isolates from healthy individuals in the same areas (**Supplementary Data**), indicating that *sasX*-positive clones spread predominantly within the hospital setting. Finally, we did not detect ST239 clones among community isolates (**Supplementary Data**), indicating that this sequence type is linked to hospital infections. Analysis of genes surrounding *sasX* in randomly selected clones of different sequence types showed that the horizontal transfer of *sasX* always occurs linked to the ΦSPβ-like prophage with a conserved phage insertion site, although occasional deletions within the phage were observed (**Supplementary Table 1**). Together, these findings demonstrate an epidemic wave of hospital-associated *sasX*-positive MRSA in China. Furthermore, they indicate events of horizontal gene transfer resulting in the spread of *sasX* from ST239 to other sequence types and a causative link between the presence of *sasX* and invasiveness.

To understand the link between *sasX* and infection, we set out to elucidate the biological role of *sasX*. We first demonstrated that the SasX protein is expressed at the *S. aureus* cell surface by immunofluorescence and immunoblot detection (**Fig. 2a,b**). Surface proteins in *S. aureus* fulfill a variety of functions that include first and foremost adhesion to host tissue[16]. In particular, adhesion to nasal epithelial cells is of major importance for the colonization, spread and virulence potential of *S. aureus* strains, inasmuch as the nares are the predominant location of *S. aureus* colonization in the human body[17] and there is an epidemiological link between nasal carriage and infection[18]. However, the molecular basis of nasal colonization is incompletely understood, and there are only a few molecular factors of *S. aureus* that have been identified as potentially responsible for nasal colonization, such as teichoic acids[19] and specific surface proteins[20–22]. To measure the impact of *sasX* on nasal colonization, we constructed isogenic *sasX* deletion mutants in representative (**Supplementary Fig. 2**) ST239 and ST5 strains. Adhesion of the *sasX*-mutant strains to human nasal epithelial cells *in vitro* was significantly impaired compared to the wild-type strains, and adhesion was restored in *sasX*-complemented mutant strains (**Fig. 2c**). Furthermore, recombinant SasX protein blocked adhesion of *S. aureus* to human nasal epithelial cells (**Fig. 2d** and **Supplementary Fig. 3**). Moreover, *sasX* had a significant impact on *in vivo* nasal colonization in separate and competitive colonization models (**Fig. 2e**). These data demonstrate that the SasX surface protein promotes nasal colonization.

In addition to facilitating adhesion to host tissue, *S. aureus* surface proteins may cause intercellular bacterial aggregation, a phenotype with multiple consequences such as increased biofilm formation and immune evasion capacity[23–25]. Indeed, we found that *sasX* facilitated the formation of large bacterial aggregates (**Fig. 3a** and **Supplementary Fig. 4**) and promoted biofilm formation (**Fig. 3b**).

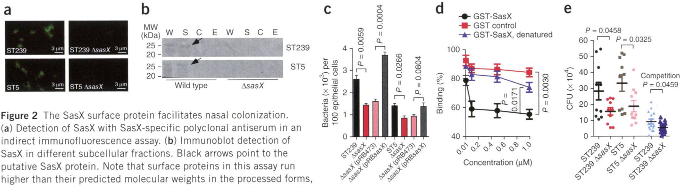

**Figure 2** The SasX surface protein facilitates nasal colonization. (**a**) Detection of SasX with SasX-specific polyclonal antiserum in an indirect immunofluorescence assay. (**b**) Immunoblot detection of SasX in different subcellular fractions. Black arrows point to the putative SasX protein. Note that surface proteins in this assay run higher than their predicted molecular weights in the processed forms, owing to the linkage to remaining peptidoglycan parts. W, whole cell preparation; S, surface protein fraction; C, cytoplasmic fraction; E, extracellular fraction. (**c**) Adherence of wild-type, isogenic *sasX*-mutant, *sasX*-complemented and control strains to human nasal epithelial cells *in vitro*. (**d**) Blocking of adherence to nasal epithelial cells by recombinant GST-SasX fusion protein. Purified GST protein alone and denatured GST-SasX fusion protein were used as controls. (**e**) Nasal colonization model. Female imprinting control region (ICR) mice ($n = 10$ per group in the separate model; $n = 15$ per group in the competition model) were inoculated intranasally with $1 \times 10^7$ colony-forming units (CFUs) of wild-type or isogenic *sasX*-mutant strains, and bacteria were counted in mice that were killed at 7 d after inoculation. ST239 HS770 was used for the separate and ST239 HS663 for the competition model. Equal amounts of wild-type and deletion mutant bacteria were mixed for the competition model inoculum. Statistical analyses in **c**–**e** are by unpaired *t* tests. All error bars show s.e.m.

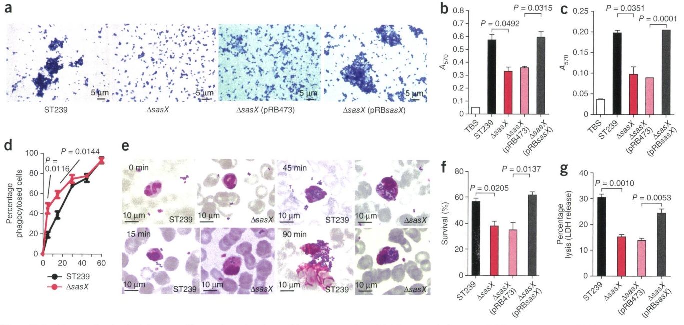

**Figure 3** SasX promotes bacterial aggregation and mechanisms of immune evasion. (**a**) Microscopic images of 16-h cultures of ST239 wild-type, isogenic *sasX*-mutant, *sasX*-complemented and control strains. (**b**) Biofilm formation. Cultures were grown for 24 h in microtiter plates. Afterward, biofilms were measured using crystal violet staining. TBS, Tris-buffered saline (buffer control). (**c**) Primary attachment. The same experiment was performed as in **b**, but with only 1 h growth. (**d**) Phagocytosis assay. Phagocytosis by human neutrophils of ST239 or the isogenic *sasX*-mutant strain was examined using microscopy. (**e**) Microscopic examination of phagocytosis. Slides were stained using a modified Wright-Giemsa stain. At $t = 90$ min, lysed neutrophils were occasionally detected in the ST239 wild-type samples. The 90-min ST239 image shows such a lysed neutrophil with adherent *S. aureus* bacteria. (**f**) Survival in human blood at $t = 90$ min. (**g**) Neutrophil killing. Bacteria from mid-logarithmic growth phase were washed and incubated with human neutrophils at a 100:1 ratio. Neutrophil lysis was determined by measuring release of lactate dehydrogenase (LDH). Statistical analyses in **b**–**d**, **f**, **g** are by unpaired *t* tests. All error bars show s.e.m.

Notably, *sasX* also facilitated primary attachment to an abiotic surface (**Fig. 3c**), which is in keeping with our finding that *sasX* expression was highest during early growth and decreased afterward (**Supplementary Fig. 5**). Moreover, phagocytosis of the wild-type strain by human neutrophils was significantly lower than that of the *sasX*-mutant strain (**Fig. 3d,e**). Accordingly, survival in human blood (**Fig. 3f**) and lysis of human neutrophils as a sign of prolonged bacterial survival after phagocytosis (**Fig. 3g**) were significantly increased in the wild-type strain compared to the *sasX* deletion mutant. Notably, these effects are most likely caused predominantly by the aggregation phenotype and not by a gene regulatory effect, as *sasX* did not affect expression of the *ica* genes encoding biofilm exopolysaccharide biosynthesis, the biofilm and virulence regulator *agr*[26] or a series of cytolysins and other surface protein genes (**Supplementary Fig. 2**). Together, these data show that *sasX* facilitates intercellular aggregation, leading to a marked enhancement of *S. aureus* immune evasion mechanisms.

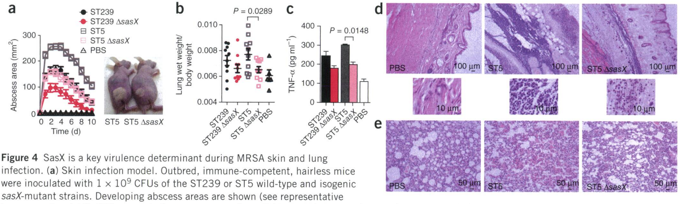

**Figure 4** SasX is a key virulence determinant during MRSA skin and lung infection. (**a**) Skin infection model. Outbred, immune-competent, hairless mice were inoculated with $1 \times 10^9$ CFUs of the ST239 or ST5 wild-type and isogenic *sasX*-mutant strains. Developing abscess areas are shown (see representative images on the right). Differences between corresponding wild-type and mutant abscess sizes were statistically significant at every time point (unpaired *t* tests, $P < 0.05$). (**b**) Lung infection model. Female ICR mice were inoculated in the nose with $1 \times 10^9$ CFUs per 20 µl of the ST239 or ST5 wild-type or isogenic *sasX*-mutant strains. Mice were killed at day 5 after infection for subsequent analyses. Lung wet weight/body weight ratios are shown. (**c**) Lung infection model, concentration of TNF-α in lung tissue samples. (**d**) Skin infection model, H&E-stained tissue samples. (**e**) Lung infection model, H&E-stained tissue samples. Note increased number of infiltrating inflammatory cells in wild-type sample images in **d** and extensive inflammation accompanied by hemorrhagic infiltration and disruption of pulmonary architecture in wild-type sample images in **e**. Statistical analyses in **b**,**c** are by unpaired *t* tests. All error bars show s.e.m.

*S. aureus* most frequently causes skin and respiratory infections[27]. Correspondingly, these were also the most abundant types of infection detected in our study (**Supplementary Fig. 6**). Among infections caused by *sasX*-positive clones, respiratory and pleural-cavity infections as well as abscesses were most prominent (significant difference by type of infection, $P = 0.0139$, $\chi^2$ test). Therefore, to determine whether *sasX* is a virulence factor during *S. aureus* infection, we selected to perform mouse abscess and lung infection models (**Fig. 4**). Mice infected with the wild-type strains developed abscesses that were significantly larger than those in mice infected with the *sasX*-mutant strains (**Fig. 4a**). Furthermore, microscopic evaluation showed increased infiltration of inflammatory cells in the abscesses caused by the wild-type strain (**Fig. 4d**). Of note, the strong impact of *sasX* on abscess size is similar to that observed with potent *S. aureus* toxins such as $\alpha$-toxin[28] or phenol-soluble modulins[29]. In addition, lung wet weight/body weight ratios, degree of inflammation in the lung as assessed by tumor necrosis factor-$\alpha$ (TNF-$\alpha$) concentration, and histological examination showed that *sasX* is also a major factor influencing *S. aureus* pathogenesis in the lungs (**Fig. 4b**,**c**,**e**). Thus, our data classify *sasX* as a key determinant of MRSA skin and lung infection.

In conclusion, we identified *sasX* as a crucial factor promoting nasal colonization, immune evasion and virulence and a probable main driving force of the Asian MRSA epidemic. Furthermore, our data provide strong support to the notion that acquisition of specific molecular determinants via horizontal gene transfer represents a major mechanism enhancing the pathogenic potential and epidemiological success of MRSA clones. Moreover, given our data, it is to be expected that the frequency of *sasX* among Asian and international MRSA clones will increase further. We thus propose drug development or vaccination efforts aimed at SasX to prevent MRSA colonization and infection.

## METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

*Note: Supplementary information is available on the Nature Medicine website.*

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AUTHOR CONTRIBUTIONS

M.L., X.D., A.E.V., D.W., Y.S., Y.T., J.H. and F.Y. conducted experiments. M.L., B.A.D., Y.L. and M.O. planned and supervised experiments. M.O. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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# Leptin action through hypothalamic nitric oxide synthase-1–expressing neurons controls energy balance

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**Few effective measures exist to combat the worldwide obesity epidemic[1], and the identification of potential therapeutic targets requires a deeper understanding of the mechanisms that control energy balance. Leptin, an adipocyte-derived hormone that signals the long-term status of bodily energy stores, acts through multiple types of leptin receptor long isoform (LepRb)-expressing neurons (called here LepRb neurons) in the brain to control feeding, energy expenditure and endocrine function[2–4]. The modest contributions to energy balance that are attributable to leptin action in many LepRb populations[5–9] suggest that other previously unidentified hypothalamic LepRb neurons have key roles in energy balance. Here we examine the role of LepRb in neuronal nitric oxide synthase (NOS1)-expressing LebRb (LepRb$^{\text{NOS1}}$) neurons that comprise approximately 20% of the total hypothalamic LepRb neurons. *Nos1*$^{cre}$-mediated genetic ablation of LepRb (*Lepr*$^{Nos1\text{KO}}$) in mice produces hyperphagic obesity, decreased energy expenditure and hyperglycemia approaching that seen in whole-body LepRb-null mice. In contrast, the endocrine functions in *Lepr*$^{Nos1\text{KO}}$ mice are only modestly affected by the genetic ablation of LepRb in these neurons. Thus, hypothalamic LepRb$^{\text{NOS1}}$ neurons are a key site of action of the leptin-mediated control of systemic energy balance.**

Commensurate with the diverse processes controlled by leptin, specialized types of LepRb neurons are located in multiple brain regions that are involved in the regulation of systemic energy balance, including the brainstem, midbrain and hypothalamus[10–13]. Knockdown or deletion of LepRb in the hindbrain interferes with satiation, although these alterations have only a slight affect on body adiposity[8,9]. Within the midbrain ventral tegmental area and substantia nigra, a subset of dopamine neurons contain LepRb; leptin action through these neurons contributes minimally to body weight control but does have a role in dopamine-mediated behaviors, including those that are linked to anxiety[14–17]. Midbrain serotonin neurons, although initially reported to have a key role in leptin action, neither express LepRb nor contribute to leptin action[18,19]. In contrast, genetic ablation of hypothalamic LepRb produces a profound metabolic phenotype, showing the key role of hypothalamic LepRb signaling in leptin action[20].

Within the hypothalamus, the specific set(s) of LepRb neurons that are responsible for the control of energy balance by leptin are not completely defined. Direct leptin action through the proopiomelanocortin (Pomc)-expressing neurons (Pomc neurons) of the hypothalamic arcuate nucleus (ARC) and through the ARC agouti-related peptide (Agrp)-expressing neurons (Agrp neurons) and steroidogenic factor-1 (Sf-1)-expressing neurons of the ventromedial hypothalamic nucleus contributes only modestly to the overall energy balance[5–7]. LepRb neurons in the lateral hypothalamic area, including those that contain neurotensin, mediate the action of leptin on orexin neurons and the mesolimbic dopamine system, but genetic deletion of LepRb from these neurons only modestly increases adiposity[21–23]. Thus, the identity of the hypothalamic LepRb neurons that are responsible for the majority of leptin-mediated regulation of energy balance is unclear.

LepRb$^{\text{NOS1}}$ neurons comprise a relatively small population of LepRb neurons and are primarily restricted to the hypothalamus, where they are distributed in areas in which they are poised to affect output from the paraventricular hypothalamic nucleus (signaling by this nucleus mediates much of the hypothalamic control of energy balance)[24–26]. To study LepRb$^{\text{NOS1}}$ neurons, we inserted an internal ribosome entry site (IRES) plus the coding sequences for Cre recombinase into the 3′ untranslated region of *Nos1* in mice to promote Nos1-restricted Cre expression (resulting in *Nos1*$^{cre}$ mice) (**Fig. 1a**). We bred *Nos1*$^{cre}$ mice to mice from the Cre-dependent ROSA26–enhanced GFP (eGFP) reporter line, generating *Nos1*$^{eGFP}$ mice. The immunoreactivity of eGFP overlapped with the immunoreactivity of Nos1 in the soma of the *Nos1*$^{eGFP}$ mice in all regions examined (data not shown).

We next examined the leptin-stimulated induction of phosphorylated signal transducer and activator-3 (pStat3), which reveals neurons containing functional LepRb (ref. 27), and its colocalization with eGFP immunoreactivity in *Nos1*$^{eGFP}$ mice. We also evaluated the colocalization of Nos1 immunoreactivity with eGFP immunoreactivity in *Lepr*$^{eGFP}$ mice[12] that express eGFP in their LepRb neurons (**Supplementary Figs. 1** and **2**). As has been previously suggested by the colocalization of NADPH diaphorase with leptin-stimulated

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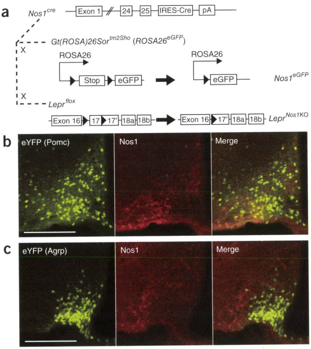

**Figure 1** Generation of $Nos1^{cre}$ and the lack of Nos1 in ARC Pomc and Agrp neurons. (**a**) Schematic diagram showing the cross (X) of $Nos1^{cre}$ with $Gt(ROSA)26Sor^{tm2sho}$ $(ROSA26^{eGFP})$ mice to generate $Nos1^{eGFP}$ mice and the cross of $Nos1^{cre}$ with $Lepr^{flox}$ mice to generate $Lepr^{Nos1KO}$ mice. pA, polyadenylation signal. The *Lepr* exons are shown, including the truncating exon 17′ that replaces exon 17 after its excision. (**b**) Representative images from the ARCs of $Pomc^{eYFP}$ mice showing enhanced yellow fluorescent protein (eYFP) (green, left), Nos1 immunoreactivity (red, middle) and merged images (right). (**c**) Representative images from the ARCs of $Agrp^{eYFP}$ mice showing eYFP (green, left), Nos1 immunoreactivity (red, middle) and merged images (right). Scale bars, 200 μm.

pStat3 immunoreactivity[24], our analysis revealed that although Nos1 and LepRb are each expressed in many brain areas alone, they are coexpressed predominantly in the hypothalamus: approximately 20% of hypothalamic LepRb neurons contain *Nos1* expression. Most LepRb$^{\text{NOS1}}$ neurons are located in the ventral premammillary nucleus (PMv), however, the dorsomedial hypothalamic nucleus (DMH) and ARC also contain a substantial number of these neurons; other areas contain few LepRb$^{\text{NOS1}}$ neurons. Nos1-expressing cells in the ARC are distinct from Pomc and Agrp neurons (**Fig. 1b**,**c** and **Supplementary Fig. 3**).

To determine the role of LepR-b$^{\text{NOS1}}$ neurons in leptin action, we crossed $Nos1^{cre}$ mice with $Lepr^{flox}$ mice[28]. Because *Nos1* is expressed in gametes, germline $Lepr^{flox}$ excision generated a *LeprΔ* allele and occurred in offspring having one $Nos1^{cre}$; $Lepr^{flox/+}$ parent. We therefore interbred $Nos1^{cre}$; $Lepr^{\Delta/+}$ and $Lepr^{flox/flox}$ mice to generate $Nos1^{cre}$; $Lepr^{\Delta/flox}$ ($Lepr^{Nos1KO}$) mice in which LepRb was ablated specifically from the LepRb$^{\text{NOS1}}$ neurons (**Fig. 1a**), as well as $Nos1^{cre}$; $Lepr^{\Delta/+}$ and $Lepr^{\Delta/flox}$ littermates to use as the normal control groups. The persistence of Cre in the early embryos of some mice also led to the production of $Nos1^{cre}$; $Lepr^{\Delta/\Delta}$ littermates that had inactivated LepRb in all tissues ($Lepr^{KO}$ mice).

An analysis of the hypothalami of control, $Lepr^{Nos1KO}$ and $Lepr^{KO}$ mice (**Supplementary Fig. 4a**,**b**) showed an absence of pStat3 in the $Lepr^{KO}$ mice. We also confirmed the ablation of leptin-stimulated pStat3 immunoreactivity from the PMvs of $Lepr^{Nos1KO}$ mice, along with modestly lower pStat3 immunoreactivity in other areas (specifically, the ARC and DMH) that contained LepRb$^{\text{NOS1}}$ neurons as compared to controls. Thus, functional LepRb was ablated from the LepRb$^{\text{NOS1}}$ neurons of $Lepr^{Nos1KO}$ mice. Consistent with the lack of overlap in the immunoreactivity between Pomc and Nos1 neurons (**Fig. 1b** and **Supplementary Fig. 3**), treatment with leptin stimulated pStat3 immunoreactivity of ARC Pomc neurons in similar amounts in control and $Lepr^{Nos1KO}$ mice (**Supplementary Fig. 4c**).

Male $Lepr^{Nos1KO}$ and $Lepr^{KO}$ mice had similarly high body weight and food intake relative to controls (**Fig. 2a**,**b**). The maximal oxygen consumption ($VO_2$) in male $Lepr^{Nos1KO}$ mice was lower than in controls but was not different from the $Lepr^{KO}$ mice; we observed similar trends for ambulatory activity (**Fig. 2c**,**d** and **Supplementary Fig. 5**). The excess weight in the male $Lepr^{Nos1KO}$ and $Lepr^{KO}$ mice was largely caused by higher adipose mass (**Fig. 2e**), which was reflected by higher leptin concentrations, relative to controls (**Fig. 2f**). We observed similar trends in female $Lepr^{Nos1KO}$ mice (**Supplementary Fig. 6**), although the obesity and metabolic dysfunction in these mice was less severe than in female $Lepr^{KO}$ mice. Thus, direct leptin action on LepRb$^{\text{NOS1}}$ neurons is crucial for the regulation of feeding, activity and energy expenditure and, therefore, the control of body weight and adiposity.

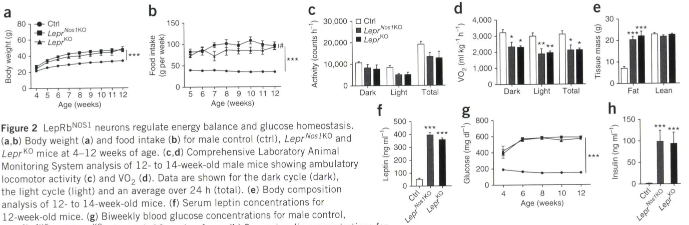

**Figure 2** LepRb$^{\text{NOS1}}$ neurons regulate energy balance and glucose homeostasis. (**a**,**b**) Body weight (**a**) and food intake (**b**) for male control (ctrl), $Lepr^{Nos1KO}$ and $Lepr^{KO}$ mice at 4–12 weeks of age. (**c**,**d**) Comprehensive Laboratory Animal Monitoring System analysis of 12- to 14-week-old male mice showing ambulatory locomotor activity (**c**) and $VO_2$ (**d**). Data are shown for the dark cycle (dark), the light cycle (light) and an average over 24 h (total). (**e**) Body composition analysis of 12- to 14-week-old mice. (**f**) Serum leptin concentrations for 12-week-old mice. (**g**) Biweekly blood glucose concentrations for male control, $Lepr^{Nos1KO}$ and $Lepr^{KO}$ mice at 4–12 weeks of age. (**h**) Serum insulin concentrations for 8-week-old mice. All data are means ± s.e.m. $n \geq 8$ for all measurements. $^{*}P < 0.05$ compared to control, $^{**}P < 0.01$ compared to control, $^{***}P < 0.001$ compared to control, $^{\#}P < 0.05$. The *P* values were not significant for all other comparisons. Statistical significance was determined by analysis of variance (ANOVA).

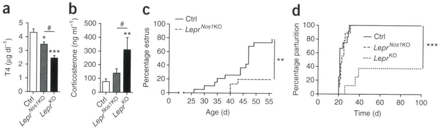

**Figure 3** LepRb$^{\mathrm{NOS1}}$ neurons contribute modestly to endocrine functions. (**a**,**b**) Serum T4 (**a**) and corticosterone (**b**) concentrations from 7- to 8-week-old male control, *Lepr*$^{Nos1KO}$ and *Lepr*$^{KO}$ mice. $n \geq 5$ for all measurements. Data are means ± s.e.m. (**c**) Analysis of time to first estrus for female control and *Lepr*$^{Nos1KO}$ mice. The percentage of mice showing vaginal estrus is plotted by age. $n \geq 9$ for all measurements. (**d**) Analysis of reproductive competence in female control, *Lepr*$^{Nos1KO}$ and *Lepr*$^{KO}$ mice. The percentage of mice delivering pups by days after mating is plotted. $n \geq 8$ for all measurements. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control, $^{\#}P < 0.05$. The *P* values for all other comparisons were not significant. Statistical significance was determined by ANOVA.

To determine the potential role for leptin action through LepRb$^{\mathrm{NOS1}}$ neurons in glucose homeostasis, we examined circulating blood glucose and insulin concentrations in control, *Lepr*$^{Nos1KO}$ and *Lepr*$^{KO}$ mice (**Fig. 2g,h**). As with body weight and adiposity, male *Lepr*$^{Nos1KO}$ and *Lepr*$^{KO}$ mice had similarly high blood glucose concentrations relative to controls starting at weaning, despite having high insulin concentrations relative to controls. Thus, leptin action through LepRb$^{\mathrm{NOS1}}$ neurons is indispensible for the normal regulation of glucose homeostasis in addition to its role in energy balance, and LepRb$^{\mathrm{NOS1}}$ neurons are crucial regulators of metabolic leptin action.

To investigate the contribution of LepRb$^{\mathrm{NOS1}}$ neurons to the control of endocrine systems by leptin, we examined circulating hormone concentrations and parameters of reproductive function in control, *Lepr*$^{Nos1KO}$ and *Lepr*$^{KO}$ mice (**Fig. 3**). Although the circulating T4 concentrations in male *Lepr*$^{Nos1KO}$ mice were lower than in controls, they were higher than in male *Lepr*$^{KO}$ mice (**Fig. 3a**). Furthermore, although circulating corticosterone concentrations were higher in male *Lepr*$^{KO}$ mice than in male *Lepr*$^{Nos1KO}$ and control mice, the circulating concentrations of corticosterone were not different between male *Lepr*$^{Nos1KO}$ and control mice (**Fig. 3b**). The onset of vaginal estrus was delayed relative to controls in singly housed female *Lepr*$^{Nos1KO}$ mice (**Fig. 3c**); however, after mating with male C57BL/6 mice, all female *Lepr*$^{Nos1KO}$ mice delivered litters with similar timing as controls (control, 23 ± 1 d (mean ± s.e.m. for time from mating to delivery); *Lepr*$^{Nos1KO}$, 24 ± 1 d) and with similar litter sizes (control, 6.9 ± 1.1 pups per litter (mean ± s.e.m.); *Lepr*$^{Nos1KO}$, 7.1 ± 0.7 pups) (**Fig. 3d**). In contrast, the majority of the female *Lepr*$^{KO}$ mice failed to deliver pups within 6 weeks of mating.

Thus, although deletion of LepRb from LepRb$^{\mathrm{NOS1}}$ neurons promoted obesity and metabolic dysfunction to an extent similar to that observed in mice with LepRb deleted throughout the body, the control of the thyroid and adrenal axes remained relatively intact, and female reproductive function was partially affected. Hence, our findings reveal that leptin action through LepRb$^{\mathrm{NOS1}}$ neurons contributes modestly to the control of endocrine function but is indispensible for the proper control of energy balance and glucose homeostasis.

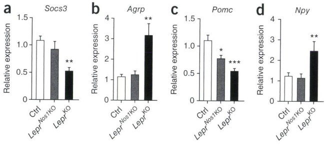

**Figure 4** Gene expression in the ARCs of *Lepr*$^{Nos1KO}$ mice. (**a**–**d**) *Socs3* (**a**), *Agrp* (**b**), *Pomc* (**c**) and *Npy* (**d**) mRNA expression by quantitative PCR (qPCR) of microdissected ARC samples from male and female control, *Lepr*$^{Nos1KO}$ and *Lepr*$^{KO}$ mice at 7–8 weeks of age that were fed *ad libitum*. $n \geq 14$ for all measurements. All data are means ± s.e.m. $^{*}P < 0.05$ compared to control, $^{**}P < 0.01$ compared to control, $^{***}P < 0.001$ compared to control. The *P* values for all other comparisons were not significant. Statistical significance was determined by ANOVA.

Relatively few ARC LepRb neurons express Nos1, and the deletion of LepRb from LepRb$^{\mathrm{NOS1}}$ neurons was not sufficient to detectably alter the expression of *Socs3* in the ARCs (a surrogate for ARC LepRb signaling[29]) of control, *Lepr*$^{Nos1KO}$ and *Lepr*$^{KO}$ mice (**Fig. 4a**). We examined the expression of key neuropeptides that regulate food intake in these mice. Although the expressions of *Npy* and *Agrp* were the same in *Lepr*$^{Nos1KO}$ mice as in controls, *Pomc* expression was lower in *Lepr*$^{Nos1KO}$ mice than in controls (**Fig. 4b–d**), even though LepRb$^{\mathrm{NOS1}}$ neurons are distinct from Pomc neurons (**Fig. 1b** and **Supplementary Figs. 3** and **4c**). Thus, LepRb$^{\mathrm{NOS1}}$ neurons may indirectly mediate aspects of melanocortin action, which is consistent with recent findings revealing that leptin action through non-Pomc neurons controls key aspects of Pomc neuron function and the regulation of energy balance[5,30]. Given the severity of the metabolic defects shown by *Lepr*$^{Nos1KO}$ mice, LepRb$^{\mathrm{NOS1}}$ neurons may act by other mechanisms as well.

Thus, leptin action through LepRb$^{\mathrm{NOS1}}$ neurons is crucial for the control of energy balance and metabolism and for the regulation of Pomc expression in the ARC Pomc neurons. Although the frequency of *Nos1*$^{cre}$-mediated excision in gametes dictated that we study mice on the heterozygous *Lepr*$^{\Delta/flox}$ background, *Lepr*$^{\Delta/flox}$ mice express the wild-type receptor on all LepRb neurons and have no detectable phenotype; it is therefore unlikely that this genetic background contributed substantially to the notable phenotype of the *Lepr*$^{Nos1KO}$ mice. Whereas *Nos1* expression identifies LepRb$^{\mathrm{NOS1}}$ neurons that are crucial for metabolic control, Nos1 signaling probably does not mediate downstream leptin action; although leptin regulates the phosphorylation and, presumably, the activity of Nos1 in hypothalamic regions containing LepRb$^{\mathrm{NOS1}}$ neurons[24], nitric oxide is a retrograde transmitter[31], and mice null for *Nos1* show no obvious primary metabolic phenotype[32]. It is therefore probable that other transmitters in the LepRb$^{\mathrm{NOS1}}$ neurons have crucial roles in properly regulating energy balance and metabolism and will be the focus of future studies.

Recent data have suggested that LepRb neurons that produce γ-aminobutyric acid (GABAergic neurons, which also express vesicular GABA transporter (vGat)), representing many of the LepRb neurons in the ARC, DMH and lateral hypothalamic area (and approximately 60–75% of the total hypothalamic LepRb neurons) are crucial for the proper control of energy balance. Approximately 20–30% of ARC and DMH LepRb$^{\mathrm{NOS1}}$ neurons contain Gad1 (which produces GABA), and a similar fraction of ARC and DMH LepRb$^{\mathrm{NOS1}}$

neurons are activated by leptin (**Supplementary Fig. 7**). By itself, direct leptin action on glutamatergic (vesicular glutamate transporter 2 (vGlut2) expressing) LepRb neurons, which comprise essentially all the LepRb neurons in the PMv and ventromedial hypothalamic nucleus, contributes only modestly to the control of energy balance by leptin[30]. Leptin activates the vast majority of PMv LepRb neurons (most of which are LepRb$^{NOS1}$ neurons)[25,33], and a variety of data support a role for PMv LepRb$^{NOS1}$ neurons (~70% of the total LepRb$^{NOS1}$ neurons) in fertility, which is consistent with the delayed estrus observed in female *Lepr*$^{Nos1KO}$ mice[34]. Because the PMv is a sexually dimorphic nucleus that is rich in androgen receptor[35], sex-specific differences in LepRb neurons in this nucleus could contribute to the sexual dimorphism observed in the *Lepr*$^{Nos1KO}$ phenotype. The apparently minor role of glutamatergic LepRb neurons in energy balance suggests that PMv LepRb$^{NOS1}$ neurons alone do not mediate the majority of the metabolic phenotype shown by the *Lepr*$^{Nos1KO}$ mice, suggesting either that the modest number of LepRb$^{NOS1}$ neurons elsewhere (for example, in the DMH and ARC) mediate this notable phenotype or that the PMv LepRb neurons reinforce the action of the DMH and ARC LepRb$^{NOS1}$ neurons by supporting action on similar neural networks. It will be crucial to understand the detailed mechanisms of action for LepRb$^{NOS1}$ neurons in the future, as this small group of cells controls energy balance and is thus a potential therapeutic target for obesity and related diseases.

## METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

*Note: Supplementary information is available on the Nature Medicine website.*

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AUTHOR CONTRIBUTIONS

R.L.L., M.G.-Y., C.M.P. and I.E.G. carried out the experiments (with staff of the Core facilities and other technical assistance). R.L.L., M.G.-Y. and C.M.P. analyzed and prepared data for publication. M.G.M. guided the overall approach, in collaboration with R.L.L. and M.G.-Y. M.G.M., R.L.L. and M.G.-Y. cowrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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TECHNICAL REPORTS

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# Unbiased identification of target antigens of CD8$^+$ T cells with combinatorial libraries coding for short peptides

Katherina Siewert[1,2], Joachim Malotka[2], Naoto Kawakami[2], Hartmut Wekerle[2], Reinhard Hohlfeld[1,2] & Klaus Dornmair[1,2]

**Cytotoxic CD8$^+$ T cells recognize the antigenic peptides presented by class I major histocompatibility complex (MHC) molecules. These T cells have key roles in infectious diseases, autoimmunity and tumor immunology, but there is currently no unbiased method for the reliable identification of their target antigens. This is because of the low affinities of antigen-specific T cell receptors (TCR) to their target MHC-peptide complexes, the polyspecificity of these TCRs and the requirement that these TCRs recognize protein antigens that have been processed by antigen-presenting cells (APCs). Here we describe a technology for the unbiased identification of the antigenic peptides presented by MHC class I molecules. The technology uses plasmid-encoded combinatorial peptide libraries and a single-cell detection system. We validated this approach using a well-characterized influenza-virus–specific TCR, MHC and peptide combination. Single APCs carrying antigenic peptides can be detected among several million APCs that carry irrelevant peptides. The identified peptide sequences showed a converging pattern of mimotopes that revealed the parent influenza antigen. This technique should be generally applicable to the identification of disease-relevant T cell antigens.**

T cell–mediated immunity has a fundamental role in health and disease, but few target antigens of tissue-infiltrating pathogenic T cells or of protective human T cells have been identified. Identification of these antigens is required for antiviral and antimicrobial diagnostics and therapeutics[1,2], for the selective immunotherapy of tumors[3,4], as well as for the development of diagnostic and therapeutic tools to treat T cell–mediated autoimmune diseases[5], such as multiple sclerosis[6,7].

A few T cell antigens have been identified by generating T cell lines from blood against *bona fide* candidate antigens, but it is unclear whether such T cell lines are representative of the T cells that mediate the immune reaction in affected tissue. A much more general approach would be to first identify T cells of interest directly in the tissue of patients and then identify the target antigens of these T cells. Antigen-reactive tissue-infiltrating T cells can be distinguished from bystander T cells by their morphological location near potential targets, their expression of activation markers and the evidence indicating their clonal expansion. These criteria can be tested using immunohistochemistry or an analysis of the relevant transcripts. From single T cells isolated by laser microdissection from frozen biopsy or autopsy tissue, the matching TCR α and β chains can then be cloned and expressed *in vitro*. Such 'resurrected' T cells can then be used in antigen searches[8].

There is currently no generally applicable and unbiased method for identifying T cell antigens, however, a number of antibody ligands have been described. This is because of several special features of TCR molecules: first, the affinities of TCRs to MHC-peptide complexes are lower than the typical affinities of antibodies to their ligands by several orders of magnitude. This precludes biochemical techniques that rely on high affinities. Second, most TCRs are polyspecific[9], meaning they can be activated not only by the parent peptide but also by peptides with up to several amino acid exchanges (termed 'mimotopes'). Randomized synthetic peptide libraries[10] use these 'motif-recognition' properties of TCR molecules to describe this polyspecificity in quantitative terms[11]. These libraries have revealed peptide mimics of CD4$^+$ T cells that are shared between humans and *Borrelia*[12], but they are limited by the fact that the few activating peptides contained in such a library are highly diluted by many irrelevant peptides and, unlike plasmids, cannot be recovered by cloning. Third, in contrast to antibodies that bind their ligands directly, TCRs recognize antigenic MHC-peptide complexes generated by a complex intracellular antigen-processing machinery[13–15]. Although complementary DNA (cDNA) libraries have helped to identify antigens from tumors and a mouse autoimmune model[16–19], their use is limited because they must contain full-length in-frame cDNA, this cDNA should originate from affected tissue, which is not always available, and correct antigen processing is required. Thus, the processing pattern of the APC used *in vitro* must be identical to that of the original APCs of the target tissue, an assumption that cannot be taken for granted. Another method uses randomized peptide libraries displayed by insect cells, which are screened by oligomerized soluble TCR molecules[20]. This approach is limited by the notoriously low affinities of TCRs to their MHC-peptide ligands and by complicated library cloning procedures. For these reasons, only a very few a priori unknown target antigens of human T cells have been discovered.

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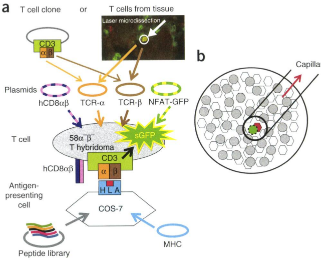


**Figure 1** Schematic overview of the experimental strategy. (**a**) $58\alpha^-\beta^-$ T hybridoma cells (gray filled oval) and COS-7 cells (light blue filled hexagon) were transfected with several expression plasmids coding for different proteins. The plasmids are shown as non-filled ovals. $58\alpha^-\beta^-$ cells were transfected with the TCR α and β chains (orange and brown), which may originate from T cell lines or clones (upper left), as was the case for the model TCR JM22 used here, or from T cells isolated by laser-microdissection from a patient's frozen tissue specimen[8] (upper right, the white arrow points to a cell to be microdissected, as indicated by a circle). This leads to the expression of TCR α and β chains (orange and brown squares) together with CD3 molecules on the surfaces of the $58\alpha^-\beta^-$ cells. Further, the human CD8 α and β chains (hCD8αβ, hatched pink and dark blue) and sGFP under the control of the NFAT enhancer (NFAT-GFP, hatched yellow and green) were transfected. For the antigen-presenting cells, we used COS-7 cells that were transiently cotransfected with the appropriate HLA molecule (blue, lower right) and with a plasmid-encoded combinatorial peptide library (multicolored, lower left). This led to the expression of HLA molecules (blue square) on the surfaces of the COS-7 cells that had bound antigenic peptides (red). This complex was recognized by the TCR transfectants, which are triggered to synthesize sGFP (yellow and green asterisk). Consequently, the TCR-transfected cells lit up green. (**b**) Coculture of transfectants allowed for the identification of the antigenic peptides. COS-7 cells transfected with MHC molecules and the library were grown in a tissue culture dish and overlaid with TCR transfectants. A COS-7 cell that expressed a peptide mimotope, which was recognized by the TCR, is indicated in red. COS-7 cells that express irrelevant peptides are shown in blue. TCR-transfected hybridoma cells that were in direct contact with a mimotope-presenting COS-7 cell were activated and lit up green. Single activated green TCR-transfected T hybridoma cells are indicated. They may denote the position of an APC that presents the correct peptide from the library. Such pairs of cells can be isolated using a capillary connected to a micromanipulator under a fluorescence microscope. From the isolated COS-7 cell, the plasmid coding for the mimotope can be cloned and sequenced.

Here we describe a generally applicable unbiased technology for the identification of target antigens of $CD8^+$ T cells. This method is extremely sensitive because single cells serve as readouts, and several million library peptides can be examined within a few hours. The approach depends neither on correct antigen processing, because short peptides are used, nor on high TCR affinity, because the cells are kept in tight physical contact by gravity.

## RESULTS

Our method combines recombinantly engineered APCs and T cells with a highly sensitive single-cell detection system. By cotransfecting a plasmid-coded combinatorial nonamer peptide library and class I MHC cDNA into COS-7 cells, which were used as APCs here, we circumvented the intricacies of cell-specific protein processing (**Fig. 1a**). In parallel, we cotransfected reporter T hybridoma cells with TCR, CD8 α and β chains[8] and super GFP (sGFP) controlled by the nuclear factor of activated T cells (NFAT)[21,22]. When seeded on top of a layer of COS-7 APCs, only the TCR transfectants that are in contact with a COS-7 cell presenting the correct antigen will light up green (**Fig. 1b**). Thus, activated TCR hybridoma cells highlight the subjacent APCs, which may then be isolated using a capillary pipette under a fluorescent microscope. The plasmid that codes for the antigenic peptide may be isolated by subcloning (**Supplementary Fig. 1**) and, eventually, sequenced.

We tested this approach using the well-investigated paradigmatic TCR JM22 (refs. 23–25), which is specific for *HLA-A*0201* (HLA-A2) and for the peptide representing amino acids 58–66 of the influenza matrix protein (flu(58–66)). We used the T hybridoma cell line $58\alpha^-\beta^-$, which lacks endogenous TCR α and β chains[26], as the recipient line for JM22, human CD8 α and β chains and sGFP controlled by NFAT (termed here 58-JM22-CD8-sGFP cells). To test whether green sGFP fluorescence can be induced in the reporter 58-JM22-CD8-sGFP cells by MHC-peptide complexes, we stably transfected COS-7 cells with HLA-A2 (COS-A2$^{\text{stable}}$) and then added the synthetic peptide flu(58–66). When we overlaid a layer of COS-A2$^{\text{stable}}$ cells with 58-JM22-CD8-sGFP cells, we observed activation of the 58-JM22-CD8-sGFP cells 16 h later (**Fig. 2a**) by detection of sGFP fluorescence under a fluorescence microscope and by using flow cytometry, the latter of which showed that 54% ± 4% (mean ± s.d.) of the cells were green. However, the fluorescence microscope detects only the brightest cells, and so the number of green cells detected using this method was lower by a factor of about three. When we used COS-7 cells lacking HLA-A2 (COS-7$^{\text{empty}}$) (**Supplementary Fig. 2a**) or replaced flu(58–66) with the irrelevant cytomegalovirus-derived peptide hCMV(184–192) (**Supplementary Fig. 2b**) in the above experiment, we did not observe any fluorescent cells among the more than 250,000 total 58-JM22-CD8-sGFP cells. This shows that 58-JM22-CD8-sGFP cells may serve as suitable readout cells to specifically detect HLA-peptide complexes at the single-cell level.

Recombinant libraries have the advantage that plasmids carrying the gene of interest can be amplified and cloned. By contrast, this is not possible with synthetic peptide or protein libraries. Therefore, we introduced the sequence coding for flu(58–66) into the expression plasmid pcDNA6/V5-HisA (pcDNA-flu(58–66)) and supertransfected COS-A2$^{\text{stable}}$ cells with this construct (**Fig. 2b**). Of the total 58-JM22-CD8-sGFP cells that we overlaid with transfected COS-A2$^{\text{stable}}$, 35% ± 2% were activated, which is only slightly lower than the proportion obtained when we used the synthetic peptide above (**Fig. 2a**). This experiment shows that short peptides encoded by pcDNA plasmids may be transported efficiently into the lumen of the endoplasmic reticula of COS-7 cells, where they are loaded onto class I MHC molecules.

Because the generation of stable HLA-transfected cells is laborious and time consuming, we cotransfected both HLA-A2 in the plasmid pRSV (pRSV-A2) and pcDNA-flu(58–66) transiently into COS-7 cells (COS-7-A2$^{\text{trans}}$). Thirty-two hours after transfection, we added 58-JM22-CD8-sGFP cells and measured the sGFP fluorescence after an additional 16 h (**Fig. 2c**). We found 14% ± 3% of

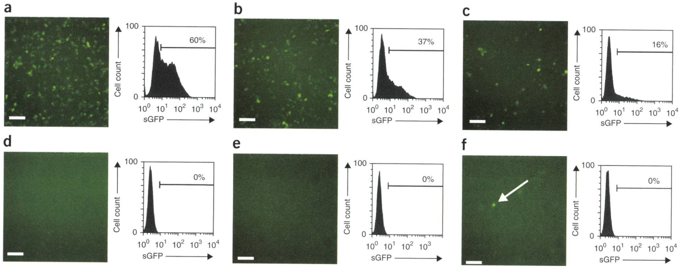


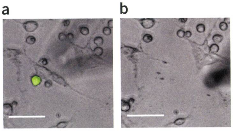
**Figure 2** Activation of TCR-transfected T hybridoma cells. Fluorescence microscopy images from cocultures of 58-JM22-CD8-sGFP cells with COS-7 cells as APCs (left images) and flow cytometry histograms of 58-JM22-CD8-sGFP cells after coculture (right graphs). The 58-JM22-CD8-sGFP cells expressed sGFP as a reporter for antigen recognition. Therefore, they lit up green on TCR activation. The percentages of activated 58-JM22-CD8-sGFP cells are indicated in the histograms. (**a**) Stably transfected COS-7-A2$^{\text{stable}}$ cells incubated with synthetic flu(58–66) peptide, yielding the maximal yield of 60% activated cells. (**b**) COS-7-A2$^{\text{stable}}$ cells transfected with the plasmid pcDNA-flu(58–66), with a yield of 37% activated cells. (**c**) COS-7 cells transiently cotransfected with HLA-A2 (COS-7-A2$^{\text{trans}}$) and pcDNA-flu(58–66), with a yield of 16% activated cells. (**d**) Non-HLA-transfected COS-7 (COS-7$^{\text{empty}}$) cells transfected with pcDNA-flu(58–66) as a control. No activated cells were observed in this group. (**e**) COS-7-A2$^{\text{trans}}$ cells cotransfected with the full-length influenza matrix protein pcDNA-flu(1–252). No activated cells were observed in this group. (**f**) COS-7-A2$^{\text{trans}}$ cells transfected with pcDNA-lib$^{\text{A2}}$, which codes for a combinatorial peptide libray. A single green 58-JM22-CD8-sGFP cell is indicated with an arrow in the fluorescence microscopy image, but the frequency of the activated cells was too low to be measured using flow cytometry. Scale bars, 100 μm. Data are representative of six independent experiments.

the 58-JM22-CD8-sGFP cells to be activated at this time point. This percentage was lower than that found when we used stable HLA transfectants (**Fig. 2b**), which can be generated when a particular MHC is used frequently. The TCR activation was specific, as 58-JM22-CD8-sGFP cells were not activated by COS-7$^{\text{empty}}$ cells transfected with pcDNA-flu(58–66) (**Fig. 2d**) or by COS-7-A2$^{\text{trans}}$ cells that were transfected with pcDNA6/V5-HisA without an insert (pcDNA-empty) (**Supplementary Fig. 2c**). Inhibition of the intracellular transport of HLA molecules with brefeldin A also abrogated the activation of 58-JM22-CD8-sGFP cells (**Supplementary Fig. 2d**). Further, activation of these cells by COS-7-A2$^{\text{trans}}$ could be blocked by the HLA-specific monoclonal antibody W6/32 (**Supplementary Fig. 2e**) but not by an isotype control antibody (**Supplementary Fig. 2f**). Activation of TCR-transfected hybridoma cells was strongly dependent on coactivation by CD8αβ molecules (**Supplementary Fig. 3**).

Full-length proteins must be degraded into short peptides before they can be loaded onto class I MHC molecules and presented to T cells. Such intricate processing steps are also required when cDNA libraries are used. We therefore tested whether the full-length influenza matrix protein flu(1–252) is processed correctly when expressed in COS-7 cells. Thus, we cotransfected pRSV-A2 and flu(1–252) cDNA in the plasmid pcDNA6/V5-HisA (pcDNA-flu(1–252)). Although flu(1–252) protein was expressed in the cytosol of the resulting COS-7 cells (**Supplementary Fig. 4**), we could not identify any green cells out of 250,000 total 58-JM22-CD8-sGFP cells (**Fig. 2e**). This shows that the COS-7 cells were indeed unable to correctly process the full-length flu(1–252) protein and clearly shows the advantage of transfecting peptides that do not need to be processed.

To simulate a scenario in which the antigenic peptide is unknown a priori, we generated combinatorial peptide libraries in pcDNA6/V5-HisA plasmid (**Fig. 1**). This plasmid (termed pcDNA-lib$^{\text{A2}}$) codes for nonameric peptides where the three known *HLA-A*0201* anchor amino acids[27] at positions 2, 6 and 9 were fixed, but all the other residues were coded by random nucleotides. We transiently cotransfected pcDNA-lib$^{\text{A2}}$ with pRSV-A2 into COS-7 cells and found subsequent activation of single 58-JM22-CD8-sGFP cells. Although the frequency of these activated cells was far too low to be detected by flow cytometry, we could detect single, green, activated 58-JM22-CD8-sGFP cells that were in direct contact with COS-7 cells using fluorescence microscopy (**Fig. 2f**). On average, we found 0.77 activated hybridoma cells per cm$^2$ of a tissue culture dish, corresponding to about 1 activated cell per 150,000 COS-7 cells. When we used a completely randomized library that did not contain any fixed anchors, we found 0.066 activated cells per cm$^2$. These notably high yields of activated cells can be explained by the fact that several different amino acids may serve as anchors and that not all anchor positions are required for HLA binding. We observed sGFP expression as early as 5 h after starting the coculture and found that it continued for more than 16 h (**Supplementary Video 1**). During the entire incubation time, activated 58-JM22-CD8-sGFP cells stayed in close contact with the underlying COS-7 cells, similar to T cell–APC interactions *in vivo*[28].

To recover plasmids coding for peptides recognized by the TCR JM22, we isolated activated 58-JM22-CD8-sGFP cells (**Fig. 3a**) together with their subjacent COS-7 cells using a capillary pipette under a fluorescence microscope (**Fig. 3b** and **Supplementary Video 2**). We then subcloned the peptide-encoding plasmids that were contained in the COS-7 cells (**Supplementary Fig. 1**), as we obtained more than one plasmid species in this initial isolation step. Possible reasons for this are that some plasmids were still present in the culture medium and were aspirated together with the cells, that we occasionally picked more than one COS-7 cell or that a single COS-7 cell can contain several different plasmids. After subcloning,



**c**

|  | 58 | 59 | 60 | 61 | 62 | 63 | 64 | 65 | 66 | Times found |
|---|---|---|---|---|---|---|---|---|---|---|
| Flu(58–66) | G | I | L | G | F | V | F | T | L |  |
| Library | X | I | X | X | X | V | X | X | L |  |
| Mimo-1 | T | I | L | G | F | V | F | T | L | 1 |
| Mimo-2 | A | I | L | G | W | V | F | T | L | 2 |
| Mimo-3 | Q | I | M | G | F | V | F | T | L | 4 |
| Mimo-4 | A | I | A | G | F | V | F | T | L | 1 |
| Mimo-5 | S | I | A | G | F | V | F | T | L | 1 |

**Figure 3** Activation of 58-JM22-CD8-sGFP cells by library-transfected COS-7 cells and the identification of mimotopes of flu(58–66). (**a**) Merged fluorescence and transmitted light microscopy images from a coculture of 58-JM22-CD8-sGFP cells and COS-7 cells that were cotransfected with the plasmid pRSV-A2 and a library encoded by the plasmid pcDNA-lib$^{A2}$. One 58-JM22-CD8-sGFP cell showed green fluorescence on activation by a COS-7 cell that presented an antigen. Scale bar, 50 μm. (**b**) The same position on the dish after isolation of the APC with a capillary pipette. Scale bar, 50 μm. (**c**) List of mimotopes (mimo-1 to mimo-5) identified for the TCR JM22 using the library pcDNA-lib$^{A2}$. The top two rows list the amino acid positions and the sequence of the parent peptide, flu(58–66). The third row shows the sequence of the combinatorial peptide library that was introduced into the APCs using the plasmid pcDNA-lib$^{A2}$. "X" indicates randomized amino acids encoded by arbitrary nucleotide sequences. The amino acids that code for the known HLA-A2–binding anchors at positions 59, 63 and 66 were fixed (highlighted in light blue). The amino acids at positions 61, 62, 63 and 65 are known to contact the TCR JM22 (ref. 24) and are highlighted in brown. The amino acids in the mimotopes that were found to deviate from the parent peptide are listed in red. Most of these deviating amino acids are clustered at positions 58 and 61, which are known not to interact with the JM22 TCR or with HLA-A2. The only exception is the exchange of Phe62 to tryptophan in the peptide mimo-2, as Phe62 is known to interact with the CDR3 regions of the TCR α and β chains. The right column lists how often each mimotope was found.

we were able to determine the sequences of the individual plasmids that activated the JM22 TCR (**Fig. 3c**). We identified five mimotopes of flu(58–66) that differed from the parent sequence at positions 58, 60 and 62. In independent experiments, we identified the mimotopes that we termed here number 2 and number 3 two and four times, respectively. Altogether, we found 9 mimotopes from 123 cells that we isolated in 12 independent experiments from a total of 9 million COS-7 cells. The amino acids at positions 58 and 60 are known to interact with neither HLA-A2 nor JM22 (ref. 24). The amino acids at positions 61, 62, 63 and 65 are known to interact with the TCR JM22. In all mimotopes, these latter amino acids are identical to those in the parent peptide, with one exception: a tolerated conservative substitution of Phe62 by tryptophan, although it is known that Phe62 interacts directly with several amino acids of the complementarity-determining region 3 of the JM22 α and β chains[24]. To test whether we could identify the parent antigen of each mimotope found here, we searched the nonredundant protein database comprising all species for homologous peptides. For four of the five different mimotopes, we identified the influenza matrix protein as the candidate parent antigen among the ten proteins with the highest scores. When we restricted the search to viruses, we found all five mimotopes to be the number one rankings.

## DISCUSSION

The identification of several mimotopes of the model TCR JM22 proves that our approach is suitable for discovering previously unknown T cell antigens. Our method has several distinct advantages: first, it is extremely sensitive, as we were able to distinguish single mimotope-expressing cells from millions of mimotope-negative cells. Therefore, libraries of very high complexity can be screened within hours. High affinities of TCRs to MHC-peptide complexes are not essential using our method because the T cells and APCs are kept in contact by gravity. With our method, even solitary plasmids can be recovered, amplified and analyzed from individual APCs. Second, our method uses immortalized cell lines that can easily be transfected and grown in unlimited amounts. This allowed us to investigate the antigens of TCRs from single pathogenic T cells recovered using laser microdissection from frozen biopsy tissue rather than relying on T cell lines from peripheral blood[8]. Third, using short peptide libraries circumvents the complexities of intracellular protein processing[13–15]. This is a great advantage over screening with cDNA libraries because antigen processing *in vitro* by engineered APCs may be completely different than antigen processing by APCs in diseased tissue. Further, these 'original' APCs may often not be available and may not even be known in some cases. Fourth, the identification of several mimotopes with converging motifs facilitates the detection of the parent peptide antigen. We used here the influenza-specific human TCR JM22, which is 'public'[29], meaning it dominates epitope-specific T cell responses in many individuals, and show that our method uncovered previously unknown mimotopes of a major human pathogenic virus. This has clear and far-reaching implications for vaccination strategies in general. Moreover, our approach may reveal additional mimotopes that are not contained in databases, such as cryptic epitopes[30], sequences containing 'altered-self' amino acids or peptides from unknown viruses.

Our new technology is ideally suited to be combined with our previously described method to 'resurrect' single T cells from archival human tissues[8]. The overall strategy would be to (i) isolate pathogenic T cells directly from tissue, (ii) clone and express their paired TCR α and β chains and (iii) characterize their antigens using the approach described here, which would reduce the highly complex *in vivo* antigen presentation and recognition to a straightforward *in vitro* reaction. When applying this method to search targets of tissue-infiltrating T cells of unknown MHC restriction, it will be necessary to test several potentially relevant HLA molecules.

To investigate the pathogenic relevance of tissue-invasive T cells in a human disease, it will be necessary to identify multiple mimotopes and their parent antigens for multiple TCRs in several patients. Further, the frequencies of the T cells carrying these specificities must be measured in patients and in appropriate controls. If such investigations reveal a convergent pattern of antigen reactivity, the candidate antigens and their relevance can be further studied using standard methodology.

In conclusion, the method described here allows for the unbiased identification of antigenic mimotopes that are recognized by CD8$^+$ T cells, such as autoaggressive T cells in autoimmune diseases, tumor-infiltrating T cells in neoplastic diseases, and antiviral and antibacterial T cells in infectious diseases[1–7]. The method offers new possibilities for the selective modulation of autoaggressive T cell clones in autoimmune diseases in which CD8$^+$ T cells are known to have prominent roles. Furthermore, precise knowledge of T cell target antigens will facilitate the development of biomarkers for the detection of viral infections and tumors, assist in the diagnosis of autoimmune diseases and offer new therapeutic options, including vaccinations against viral infections or tumors with new mimotopes or TCR gene therapy[31].

## METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

*Note: Supplementary information is available on the Nature Medicine website.*

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### AUTHOR CONTRIBUTIONS

K.S. designed and performed most of the experiments and contributed to the writing of the manuscript, J.M. contributed to cloning experiments, N.K. contributed to microscopy experiments, H.W. and R.H. supervised and supported the study and contributed to the writing of the manuscript, and K.D. conceived of, designed and supervised the research and wrote the manuscript.

### COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemedicine/.

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# A brain tumor molecular imaging strategy using a new triple-modality MRI-photoacoustic-Raman nanoparticle

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**The difficulty in delineating brain tumor margins is a major obstacle in the path toward better outcomes for patients with brain tumors. Current imaging methods are often limited by inadequate sensitivity, specificity and spatial resolution. Here we show that a unique triple-modality magnetic resonance imaging–photoacoustic imaging–Raman imaging nanoparticle (termed here MPR nanoparticle) can accurately help delineate the margins of brain tumors in living mice both preoperatively and intraoperatively. The MPRs were detected by all three modalities with at least a picomolar sensitivity both *in vitro* and in living mice. Intravenous injection of MPRs into glioblastoma-bearing mice led to MPR accumulation and retention by the tumors, with no MPR accumulation in the surrounding healthy tissue, allowing for a noninvasive tumor delineation using all three modalities through the intact skull. Raman imaging allowed for guidance of intraoperative tumor resection, and a histological correlation validated that Raman imaging was accurately delineating the brain tumor margins. This new triple-modality–nanoparticle approach has promise for enabling more accurate brain tumor imaging and resection.**

The completeness of the surgical resection is a key factor in the prognosis of patients with brain tumors[1,2]. In attempting to achieve more complete glioma resections, the surgeon encounters several hurdles, including irregular and indistinct tumor margins, as well as tumor growth adjacent to or invading crucial neurological structures[3]. A wide variety of techniques have been explored in an effort to better visualize tumor margins. For example, preoperative magnetic resonance imaging (MRI) has been used to guide stereotactic surgery, where the magnetic resonance images are used to determine the macroscopic outline of the tumor[4]. However, such methods suffer from limited spatial resolution and incongruencies between the borders outlined in the preoperative MRI and the actual tumor borders during surgery because of brain shift[5]. Intraoperative MRI usually requires the administration of gadolinium (Gd) chelates, which have a short blood half-life and therefore require repeated injections[6] and high dosages[7] and the use of which may result in inaccuracies because of surgically induced false-positive contrast enhancement[8]. Several intraoperative optical methods have been suggested that are based either on intrinsic tissue optical properties[3,9] or exogenous contrast agents[10–12]. However, these optical techniques have poor specificity because of tissue autofluorescence, limited resolution and depth of penetration, which ultimately limit the localization of the true brain tumor margins[13,14].

Photoacoustic imaging is a new technology that largely overcomes the depth and resolution limits of optical imaging. In photoacoustic imaging, light pulses excite target molecular imaging agents, causing very slight heat production and thermal expansion. This produces ultrasound waves that are recorded by an ultrasound transducer that produces a three-dimensional image of the imaging agent's distribution in living subjects[15,16]. Raman imaging, another promising and complementary optical imaging technique, can be greatly enhanced by the surface-enhanced Raman scattering (SERS) effect[17]. Because of the unique signature of the SERS spectrum, Raman imaging allows for highly specific and sensitive detection of SERS contrast agents, as well as the multiplexing of multiple agents in living subjects[17–20].

An ideal molecular imaging agent would be sequestered and retained by a tumor for a long enough period that a single injection

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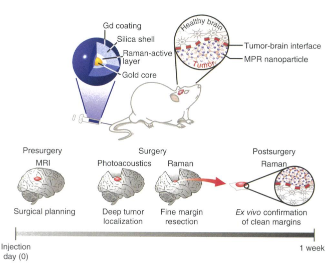

**Figure 1** Triple-modality MPR concept. MPRs are injected intravenously into a mouse bearing an orthotopic brain tumor (top). As the nanoparticles circulate in the bloodstream, they diffuse through the disrupted blood-brain barrier and are then sequestered and retained by the tumor. The MPRs are too large to cross the intact blood-brain barrier and, therefore, cannot accumulate in healthy brain. The concept of proposed eventual clinical use (bottom). Detectability of MPRs by MRI allows for preoperative detection and surgical planning. Because of the retention of the probe, only one injection is necessary, and the probe can be detected in the tumor during surgery several days later. Photoacoustic imaging, with its relatively high resolution and deep tissue penetration, is then able to guide bulk tumor resection intraoperatively. Raman imaging, with its ultrahigh sensitivity and spatial resolution, can then be used to remove any residual microscopic tumor burden. The resected specimen can subsequently be examined using a Raman probe *ex vivo* to verify clear tumor margins.

of the agent would facilitate both preoperative and intraoperative imaging, allowing for preoperative planning and intraoperative resection of the tumor. This agent should also allow for both deep tumor visualization and highly sensitive and specific detection of tumor margins.

Here we present a new approach that attempts to fulfill these criteria. We have designed and tested MPRs for a triple-modality strategy that, to our knowledge, is the first to combine MRI, photoacoustic imaging and Raman imaging. This strategy achieves (i) whole-brain tumor localization for preoperative and intraoperative macroscopic delineation using MRI, (ii) high spatial resolution and three-dimensional imaging using photoacoustic imaging and (iii) high-sensitivity, high-specificity and high-resolution surface imaging of tumor margins using Raman imaging (**Fig. 1**).

## RESULTS

### Synthesis and characterization of triple-modality MPRs

The MPR nanoparticle is composed of a 60-nm gold core covered with the Raman molecular tag *trans*-1,2-bis(4-pyridyl)-ethylene. The thin Raman-active outer layer is protected by a 30-nm silica coating. We further modified the particles with 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)-$Gd^{3+}$ using a maleimide linkage (maleimide-DOTA-Gd) (Online Methods), resulting in a gold-silica–based SERS nanoparticle coated with $Gd^{3+}$ ions (the MPR nanoparticle) (**Fig. 2a**,**b**). We determined the number of $Gd^{3+}$ ions per MPR to be 79,340 ± 2,270 (mean ± s.d.) using inductively coupled plasma atomic emission spectroscopy (ICP-AES). A portion of these $Gd^{3+}$ ions could be bound directly to the silica surface. To test the serum stability of the MPRs, we performed ICP-AES and found that the number of $Gd^{3+}$ ions per MPR decreased by only approximately 14% in the presence of mouse serum after 24 h of incubation (data not shown).

In addition, we validated that the MPRs are stable in serum by measuring their optical stability (**Supplementary Fig. 1**) and hydrodynamic size distribution (**Supplementary Fig. 2**) over the course of a 24-h incubation in mouse serum. We verified the stable binding of $Gd^{3+}$ ions to the nanoparticle surface and the absence of any free $Gd^{3+}$ ions in the solution by acquiring T1-weighted magnetic resonance images of the supernatant after particle centrifugation (data not shown). The MPRs showed a very high T1 relaxivity, $3.0 \times 10^6$ $mM^{-1}$ $s^{-1}$ (in $H_2O$ at a field strength of 7 T and at 20 °C), with minimal batch-to-batch variation (**Fig. 2c**). The MPR optical absorbance peaked at 540 nm and had a very high absorbance coefficient, $2.75 \times 10^{10}$ $cm^{-1}$ $M^{-1}$ (**Fig. 2d**). We therefore further modified our custom-made photoacoustic imaging system to include a 532-nm laser to allow for imaging of the MPRs (**Supplementary Methods**). The MPRs had a unique Raman signature (**Fig. 2e**), which was identical before and after the surface conjugation of the maleimide-DOTA-Gd to the particles (**Supplementary Fig. 3**).

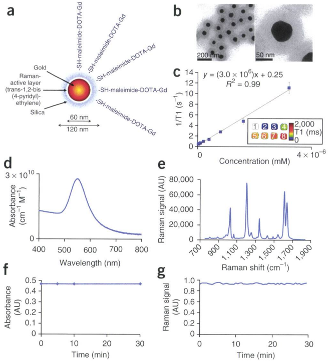

**Figure 2** Characterization of the MPRs. (**a**) Simplified diagram of the MPR. A 60-nm gold core is surrounded by a thin Raman-active layer that is protected by a 30-nm silica coating. The silica coating was further functionalized with maleimide-DOTA-Gd, which was conjugated to the thiol (SH) group on the silica. (**b**) Transmission electron microscopy images of the MPRs. (**c**) Particle relaxivity derived from T1 maps of probe dilutions in MRI phantoms. Data represent the mean of two separate phantoms containing separate probe conjugations (error bars (s.e.m.) indicate batch-to-batch variation). The inset shows a T1 map of an MRI phantom containing MPRs at concentrations ranging from 3.2 nM (1) to 25 pM (8). (**d**) Optical absorbance of the MPRs. (**e**) Raman spectrum of the MPRs, with characteristic peaks at 1,021, 1,204, 1,340, 1,614 and 1,638 $cm^{-1}$. (**f**,**g**) During 30 min of continuous laser irradiation, the optical absorbance (**f**) and the Raman signal (**g**) remained constant. AU, arbitrary units.

To test for possible photobleaching, we irradiated the MPRs using both the photoacoustic and the Raman imaging systems. During 30 min of continuous laser irradiation, the optical absorption and Raman signal each did not vary more than 2% (**Fig. 2f,g** and **Supplementary Methods**).

### *In vitro* and *in vivo* detection of MPRs

We next determined the *in vitro* detection threshold of the MPRs for each modality. We imaged an agarose phantom containing MPRs in concentrations ranging from 1.22 pM to 1,250 pM ($n = 3$ per concentration) using MRI, photoacoustic imaging and Raman imaging (**Supplementary Fig. 4a**) and determined the signal intensities using a region-of-interest analysis (**Supplementary Fig. 4b**). The lowest detectable concentrations were 4.88 pM for MRI, 1.22 pM for photoacoustic imaging and 610 fM for Raman imaging (**Supplementary Fig. 5**). The MRI, photoacoustic and Raman signals produced by the MPRs *in vitro* were highly correlated with the MPR concentrations ($P < 0.0001$ for all modalities, with $R^2 = 0.97$, 0.99 and 0.99 for MRI, photoacoustic and Raman, respectively) (**Supplementary Fig. 4b**) and were also highly linear and correlated with each other (**Supplementary Fig. 6**). For a comparison of the depth of penetration of photoacoustic imaging compared to Raman imaging, see **Supplementary Figure 7**.

We then measured the detection thresholds of the MPRs in living mice. We subcutaneously injected MPRs diluted in matrigel to six different concentrations (ranging from 50 pM to 1,100 pM) into the right flanks of nude mice ($n = 3$ mice) and scanned the mice using the MRI, photoacoustic and Raman systems (**Supplementary Fig. 8a**). The MRI, photoacoustic and Raman signals *in vivo* highly correlated to the MPR concentrations ($P = 0.001$ for all modalities, with $R^2 = 0.99$, 0.97 and 0.99 for MRI, photoacoustic and Raman, respectively) (**Supplementary Fig. 8b**). The sensitivity of the MRI and the photoacoustic imaging was limited by the intrinsic tissue background signal. For both MRI and photoacoustic imaging, 50 pM of MPRs gave an equivalent signal to muscle. Raman imaging, however, had a negligible tissue background signal and was therefore limited only by the signal-to-noise ratio. Indeed, at the nominal concentration of 50 pM, the Raman image clearly visualized the MPRs. This explains why the Raman response had the steepest slope *in vivo* as compared to those from the MRI and photoacoustic imaging ($P < 0.0001$ for Raman imaging compared to either MRI or photoacoustic imaging; however, the slope of the photoacoustic imaging was not statistically different from that of MRI, $P = 0.16$), whereas *in vitro*, where no substantial background signal was present, photoacoustic imaging had the steepest slope of the three modalities. Finally, we found a linear correlation between the signals of the three modalities (**Supplementary Fig. 9**).

### *In vivo* triple-modality brain tumor visualization of MPRs

We next sought to determine whether the MPRs could be used for orthotopic brain tumor detection in living mice. We hypothesized that in an orthotopic glioblastoma brain tumor model, the nanoparticle probe would enter the extravascular space by diffusion through the disrupted blood-brain barrier and accumulate in cells within the tumor without necessitating a specific targeting mechanism (enhanced permeability and retention (EPR) effect), as has been previously observed for iron oxide nanoparticles[22]. We used an orthotopic brain tumor model in which we implanted enhanced GFP (eGFP)-transfected human gliomablastoma cells (eGFP$^+$U87MG) into the striata of nude mice using a stereotactic implantation device (**Supplementary Methods**). We injected tumor-bearing mice ($n = 4$) through tail vein with MPRs and performed consecutive photoacoustic imaging, Raman imaging and MRI on each mouse before injection and at 2 h, 3 h and 4 h after injection, respectively (**Fig. 3** and **Supplementary Fig. 10**).

The images from after the injection demonstrated clear visualization of the tumor for all three modalities despite having been acquired through the intact skin and skulls of the mice (**Fig. 3a**). The photoacoustic and Raman images were co-registered with the MRI image, showing good colocalization between the three modalities (**Fig. 3a**). In parallel to the photoacoustic images of the brain, we also acquired co-registered ultrasound images to register the photoacoustic images to the magnetic resonance images in orthogonal planes (**Fig. 3b**) (using Amide[23] and Amira software, see **Supplementary Methods**).

A region of interest quantification of the signals in the tumors showed a significant increase in the MRI, photoacoustic and Raman signals after as compared to before the tail-vein injection (**Fig. 3c**). The MRI contrast-to-noise ratio increased from 2.2 ± 0.3 to 14.0 ± 1.9

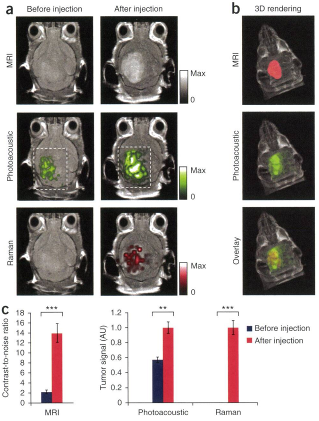

**Figure 3** Triple-modality detection of brain tumors in living mice with MPRs. (**a**) Two-dimensional axial MRI, photoacoustic and Raman images. The post-injection images of all three modalities showed clear tumor visualization (dashed boxes outline the imaged area). (**b**) A three-dimensional (3D) rendering of magnetic resonance images with the tumor segmented (red; top), an overlay of the three-dimensional photoacoustic images (green) over the MRI (middle) and an overlay of MRI, the segmented tumor and the photoacoustic images (bottom) showing good colocalization of the photoacoustic signal with the tumor. (**c**) Quantification of the signals in the tumor showing a significant increase in the MRI, photoacoustic and Raman signals after as compared to before the injection. $n = 4$ mice. Data represent mean ± s.e.m. $***P < 0.001$, $**P < 0.01$ (one-sided Student's *t* test.) AU, arbitrary units.

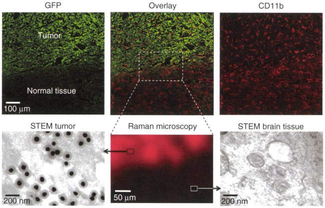

**Figure 4** Histological validation. Ten-micrometer frozen sections from the margin of an eGFP$^+$U87MG brain tumor stained for eGFP (green) to visualize the tumor margins and CD11b (red) to visualize glial cells and examined using laser scanning confocal microscopy (top). A 50-μm adjacent slice examined using Raman microscopy to visualize the distribution of the MPRs (bottom). The Raman signal corresponding to the eGFP$^+$ cells indicates the presence of the probe in the tumor but not in the adjacent healthy tissue. The Raman signal (red) was scaled from 0 to 100 AU. The boxes are not drawn to scale. STEM images verified the presence of MPRs in the brain tissue, whereas no MPRs were seen in the healthy brain tissue. A three-dimensional STEM rendering of MPRs in brain tumor is provided in **Supplementary Video 1**.

(mean ± s.e.) ($P = 0.001$). The photoacoustic signal increased by 75%, from 0.57 ± 0.02 AU to 1.0 ± 0.08 AU ($P = 0.001$). The Raman system recorded no signal before injection and an intense signal of 1.0 ± 0.09 AU ($P = 0.012$) after injection.

We then determined the kinetics of the MPRs for all three modalities using additional mice orthotopically implanted with eGFP$^+$U87MG tumor cells ($n = 4$ mice each for MRI and Raman imaging; we derived the photoacoustic data from the first set of mice described for **Fig. 3**). We analyzed the signal kinetics for each mouse individually for the MRI, photoacoustic and Raman imaging systems (**Supplementary Fig. 11**). We acquired data before injection and at 0.5 h, 1 h, 1.5 h and 2 h after injection. For MRI, we also acquired data 24 h after injection. We found that the MPR signal increased markedly between the pre-injection and the 30-min post-injection time points for all three modalities (from 1.4 ± 0.24 to 8.7 ± 0.76 contrast-to-noise ratio for MRI ($P < 0.001$), a 60% ± 14% increase for photoacoustic imaging ($P < 0.01$) and from 0 to 1.96 ± 0.27 AU for Raman imaging ($P < 0.001$)). The signal then reached a plateau for MRI and photoacoustic imaging, which remained essentially stable until the latest examined time point of 2 h for photoacoustic imaging and 24 h for MRI. Of note, this behavior contrasts with conventional clinically used Gd-based contrast agents, which show rapid washout within minutes after injection, whereas the MPRs showed persistent signal enhancement (**Supplementary Fig. 11**). For Raman imaging, we observed an initial signal peak before the signal reached a plateau ($P < 0.0001$ for the peak value compared to the plateau). We presumed this effect was caused by the initial nonspecific circulation of MPRs in superficial layers (for example, skin), to which Raman imaging is most sensitive of the three modalities (**Supplementary Fig. 11**).

## Histological validation of MPR sequestration by brain tumors

We next examined the distribution of the MPRs within the brain using histology. Using the mice described above, we performed immunohistochemistry with antibody staining against eGFP and CD11b to visualize eGFP$^+$U87MG tumor cells and microglia, respectively. In particular, we sampled sections that included the interface between the tumor and the surrounding brain tissue. We then examined adjacent sections using high-resolution Raman microscopy (**Supplementary Methods**) and correlated these images with the immunohistochemistry results. We observed a strong Raman signal within the tumors but not in healthy brain tissue, with the Raman signal producing a very good delineation of the actual tumor border (**Fig. 4**). Scanning transmission electron microscopy (STEM) (**Fig. 4**) further corroborated these results, showing numerous MPRs in tumor sections and none in the surrounding brain tissue (923 MPRs found within tumors in an examined volume of 57,500 μm$^3$ (average of 0.016 MPRs per μm$^3$) and no MPRs found in healthy brain in an examined volume of 12,500 μm$^3$). A three-dimensional STEM rendering of MPRs in the tumors is provided in **Supplementary Video 1**.

To further examine the ability of the MPRs to visualize not only the bulk tumor but also the margins of the invasive tumor, we used an orthotopic primary human xenograft glioblastoma mouse model (the TS543 cell line[24] grown as neurospheres). As confirmed by correlative Raman microscopy, immunohistochemistry and scanning electron microscopy, the MPRs accumulated in the infiltrating tumor margins (**Supplementary Fig. 12**). In addition, Raman imaging was able to depict finger-like tumor protrusions and even isolated microscopic tumor foci (**Supplementary Fig. 13**).

## MPRs guide brain tumor resection *in vivo*

Finally, we explored whether tumor resection along the photoacoustic and Raman signals, 24 h after intravenous injection of MPRs, could facilitate tumor resection. Initially, we tested the ability of

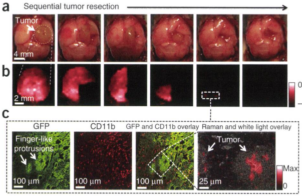

**Figure 5** Raman-guided intraoperative surgery using MPRs. (**a**,**b**) Living tumor-bearing mice ($n = 3$) underwent craniotomy under general anesthesia. Quarters of the tumor were then sequentially removed (as illustrated in the photographs, **a**), and intraoperative Raman imaging was performed after each resection step (**b**) until the entire tumor had been removed, as assessed by visual inspection. After the gross removal of the tumor, several small foci of Raman signal were found in the resection bed (outlined by the dashed white square; some Raman images are smaller than the image frame). The Raman color scale is shown in red from −40 dB to 0 dB. (**c**) A subsequent histological analysis of sections from these foci showed an infiltrative pattern of the tumor in this location, forming finger-like protrusions extending into the surrounding brain tissue. As shown in the Raman microscopy image (right), a Raman signal was observed within these protrusions, indicating the selective presence of MPRs. The box is not drawn to scale. The Raman signal is shown in a linear red color scale.

photoacoustic imaging to delineate brain tumors *in situ*, which showed a reduced signal in the resected area (**Supplementary Fig. 14**). Next, we placed brain-tumor–bearing mice ($n = 3$) under general anesthesia and performed craniotomies and subsequent *in vivo* Raman imaging. We then removed sections of the brain tumors detected using visual inspection only. We obtained high-resolution intraoperative Raman images after each resection step and correlated them with the intraoperative photographs. We first visualized the whole tumor using Raman imaging (**Fig. 5a,b**). With each sequential resection step, we noted a high congruency between residual tumor tissue (**Fig. 5a**) and the presence of a Raman signal (**Fig. 5b**) and, vice versa, between resected tumor and lack of a Raman signal. Of note, after the tumor resection seemed to be complete by visual inspection, we noted several small foci of residual Raman signal (**Fig. 5b**) in the resection bed. When we then extended the resection to include these foci, which were located near the tumor-brain interface and histologically analyzed this tissue, we found frequent finger-like microscopic extensions of the tumor into the surrounding brain tissue (**Fig. 5c**). We were able to detect these cancerous foci, which were otherwise not visible to the naked eye because of the specific accumulation of the MPRs therein.

## DISCUSSION

We designed and tested a unique triple-modality nanoparticle that is, to our knowledge, the first to combine MRI, photoacoustic and Raman imaging. The MPRs described here could enable radiologists and neurosurgeons to 'see' the same probe before and during surgery, thus allowing for more accurate brain tumor resection by exploiting the complementary strengths of each modality.

The excellent detectability of MPRs using MRI (in the picomolar range) is a direct result of the very high longitudinal relaxivity of the MPRs of $3.0 \times 10^6$ $mM^{-1}$ $s^{-1}$. To our knowledge, this is the highest relaxivity of a nanoparticle reported to date.

Photoacoustic imaging is a relatively new technique that allows deeper tissues to be imaged with higher spatial resolution compared to most optical techniques[25–27]. The exceptionally high optical absorbance coefficient of the MPRs is over 200-fold higher than, for example, previously reported photoacoustic imaging agents that are based on carbon nanotubes[28,29]. Also considering its three-dimensional capabilities, photoacoustic imaging could guide the more gross resection steps and even identify tumor tissue residing under the surface of normal brain tissue. Then, to completely remove microscopic tumor deposits, Raman imaging, with its superior sensitivity, could be used.

Raman imaging in conjunction with MPRs offers ultrahigh sensitivity in the picomolar range, as opposed to the nanomolar sensitivity that is achievable using fluorescence imaging of quantum dots[13,17,18,20]. Raman imaging of MPRs, in contrast to other optical imaging techniques, does not suffer from autofluorescence or background signal because the MPR spectral signature is highly amplified and unique (its 'fingerprint'). Although the main limitation of Raman imaging is its limited penetration depth, we achieved tumor visualization here through the intact skin and skulls of live mice (to a depth of 2–5 mm). This result stems from a combination of the design of the nanoparticle, with its gold core producing a surface plasmon resonance for Raman signal enhancement, the Raman substrate used and the number of nanoparticles accumulating within the tumor. Raman nanoparticles are inherently insensitive to photodestruction, which is a known problem of organic fluorochromes. Furthermore, unlike most quantum dots, which are cytotoxic[30,31], MPR nanoparticles are based on inert gold and silica and, thus, may have a better chance for clinical translation. Gold and gold-silica nanoparticles have excellent cytotoxicity profiles, as has been illustrated in detailed toxicity studies in cell cultures, mice[32–34] and several clinical trials[21]. The design of the MPRs would also allow for multiplexing[20], with the potential to detect multiple biomarkers simultaneously *in vivo*.

In addition, MPRs have a unique advantage over conventional contrast agents of low molecular weight. For example, low–molecular-weight Gd chelates or fluorochromes accumulate in the extracellular space, where breakdown of the blood-brain barrier has occurred, and then undergo rapid diffusion through the interstitium and renal clearance. These low–molecular-weight agents are therefore unable to delineate tumors for the time period spanning the resection procedure, let alone for the entire period between preoperative planning and surgical intervention. This diffusion process also introduces imprecision of probe localization, requires repeated contrast administration (for example, of Gd chelates during intraoperative MRI) and can cause false-positive results because of surgically induced contrast enhancement. In contrast, the *in vivo* kinetic studies performed with the MPRs here show that the particle is retained in the tumor, allowing for repeated imaging as required without the need for multiple injections. This contrast-agent behavior may also be useful for distinguishing tumor recurrence from nonspecific treatment-related effects. As the MPR approach relies on the EPR effect, it could potentially be applied to imaging other cancer types that have intrinsic EPR effects, including lung cancer, melanoma, renal cancer, hepatoma and many others[35]. In addition, the long intratumoral retention of the MPRs could also be exploited for drug delivery or photothermal therapy.

New instrumentation, including endoscopic and intraoperative photoacoustic and Raman imaging devices that are required for the clinical translation of the MPR approach described here, are currently under development[36,37]. Ideally, a combination of both devices integrated into one handheld probe would be available in the operating room. In particular, such endoscopes should be designed for easy intraoperative navigation and should enable real-time imaging. Further development of such instrumentation could lead to improved brain tumor surgery and outcomes for patients in the future. For an additional discussion, see the **Supplementary Discussion**.

## METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

*Note: Supplementary information is available on the Nature Medicine website.*

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### AUTHOR CONTRIBUTIONS

M.F.K. co-initiated the project, designed the research, synthesized and characterized MPR nanoparticles, performed MRI, Raman, photoacoustic and histology experiments, analyzed data and wrote the manuscript. A.d.l.Z. modified the photoacoustic system, designed and performed photoacoustic experiments, analyzed data and wrote the manuscript. J.V.J. synthesized and characterized MPR nanoparticles. C.L.Z. designed, performed and analyzed Raman experiments and edited the paper. P.J.K. and R.S. performed and analyzed the electron microscopy experiments. K.P. performed immunohistochemistry. F.H. helped create three-dimensional renderings. E.M., M.F.K., K.P., R.H., C.C., C.W.B., I.K.M. and E.C.H. provided mouse models. S.S.G. co-initiated the project, designed the research, analyzed data, supervised and coordinated all investigators for the project and wrote the manuscript.

### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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# Institut Curie

Paris, France

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Together, let's beat cancer.

## Call for Translational Research Team Leaders

The Institut Curie, is a private, non-profit, charitable foundation, dedicated to fundamental and cancer research as well as cancer patient care. A total of 3000 people contribute to the scientific research, cancer diagnosis and treatment and the teaching and training activities of the Institut Curie. The Institut Curie offers a unique scientific and medical environments including internationally recognized departments in immunology, cell and developmental biology, genetics and epigenetics, biophysics and chemistry as well as dedicated cancer hospitals with a strong focus on the development of innovative early phase clinical trials. The main malignancies treated in Institut Curie include breast, eye, pediatric, gastro-intestinal and gynecological cancers. Institut Curie gives access to state-of-the-art core facilities (next generation sequencing, genomics, microscopy, mass spectrometry, animal facility, automated-imaging cell-based facility...) as well as large clinical databases and sample collections.

To strengthen its translational research department and foster the links between basic research and care to patients, **the Institut Curie is seeking to recruit outstanding translational research teams in major translational research areas including molecular pathology, assessment and biology of the metastatic spread, genetic susceptibility factors, radiobiology, preclinical and early clinical trials.** Identification and validation of new biomarkers that are required for an improved diagnosis and prognostic evaluation, for a better assessment of follow-up and response to treatment particularly in the frame of targeted therapies constitute a priority.

Applicants should be **MDs with PhD or with equivalent research experience.** They are expected to contribute to patients management in the clinical departments and to spend a significant part of their time in the laboratory. Application by PhDs may be considered if excellent connection with the clinical activities can be demonstrated.

A lab space of approximately 60 square-meters and an initial support package including salary for the PI and for a post doc will be provided as well as access to technology platforms and common infrastructures.

Applications should include a CV (with list of publications and conferences), a summary of scientific and medical achievements (1-2 pages), a research plan (5 pages) and letters of recommendation.

Application should be sent to:
Dr. Olivier Delattre, Deputy Director for Biomedical Research and
Dr. Sergio Roman Roman, Head of Translationnal Research Department

Mail contact: elisabeth.hess@curie.fr

The deadline for application is: June 15th, 2012

Preselected candidates will be interviewed in October or November

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## *Heal the sick, advance the science, share the knowledge.*

**Mayo Clinic in Rochester, Minnesota** seeks outstanding candidates for research faculty positions in the field of cardiovascular regenerative medicine, a strategic priority of the institution. This is a coordinated, multidepartment recruitment in fundamental and diseased-focused areas pertinent to stem cell biology (e.g., nuclear reprogramming, developmental biology, tissue bioengineering) as they apply to the broader fields of cardiovascular (heart and vascular repair) diseases. We also encourage candidates utilizing interdisciplinary approaches relevant to human disease with expertise in regenerative diagnostics or therapeutics. Candidates should hold an MD, PhD, MD/PhD or equivalent degree and have a demonstrated record of relevant research accomplishments as evidenced by their training, publications and peer review funding. Appointment and rank in an academic department will be determined based on the applicant's qualifications.

Mayo Clinic is an internationally renowned, integrated, multidisciplinary academic medical center with comprehensive programs in medical education and research with campuses in Rochester, Minnesota; Jacksonville, Florida; and Scottsdale/ Phoenix, Arizona. Mayo Clinic supports five schools spanning undergraduate, graduate, medical, postgraduate medical and continuing medical education, and has a vibrant research enterprise with programs in clinical, basic and population sciences. In 2008, the institution received more than $325 million in extramural research awards.

Mayo Clinic's location in Rochester, MN, combines the ease of small city living with easy access to additional cultural and entertainment opportunities in nearby Minneapolis/St. Paul. Mayo Clinic offers a competitive salary, excellent benefits and has been recognized by *Fortune* magazine as one of the "100 Best Companies to Work For." To learn more about Mayo Clinic and Rochester, MN, please visit **www.mayoclinic.org/physician-jobs/**

Interested candidates are invited to submit a cover letter, curriculum vitae, brief description of research plans and three letters of reference to: **RegenerativeMedicinePositions@Mayo.edu**

**Applications will be accepted until the positions are filled.**

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**Neil Risch** (University of California, San Francisco, USA)
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**Les Biesecker**
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**Mary Carrington**
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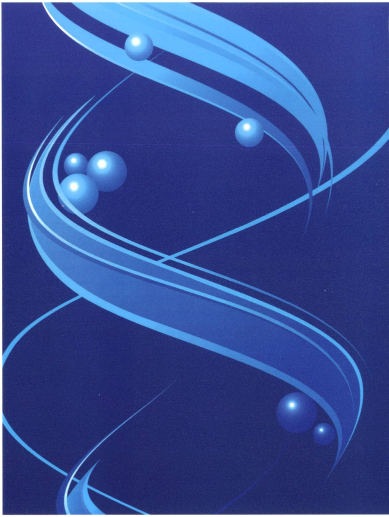

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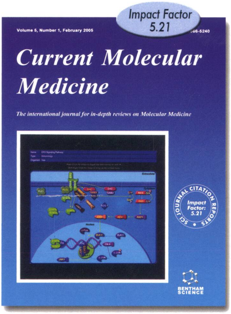
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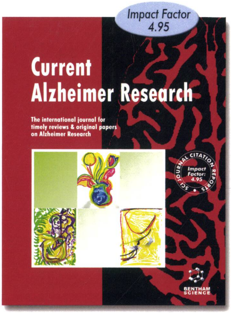
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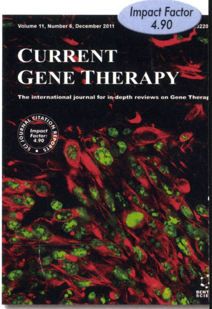
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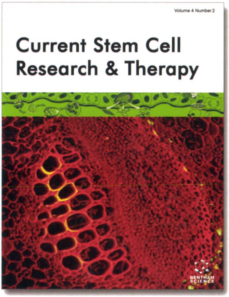
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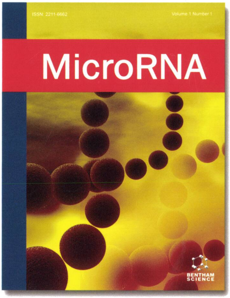
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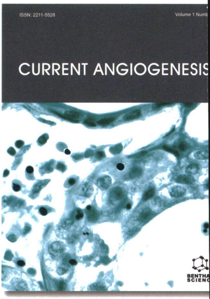
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